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SEPARATION OF PHOSPHOLIPIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND QUANTITATIVE ANALYSIS USING NON-DIGESTIVE COLORIMETRIC TECHNIQUES

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **Separation of Phospholipids by High Performance Liquid Chromatography and Quantitative Analysis Using Non-digestive Colorimetric Techniques** submitted by **George William Ruddock** in partial fulfilment of the requirements for the degree of **Master of Science** in **Food Chemistry**.

ABSTRACT

The HPLC separation of selected phospholipids (PE, LPE, PC, Spm and LPC) has been achieved on a Whatman silica gel column (500 x 4.6 mm) using an acetonitrile: methanol:water (51:35:14 by vol.) solvent system. The solvent system was developed utilizing UV absorption at 205 nm for peak identification, but this was found to be inadequate for quantification. Hence, alternative quantification systems employing both digestive and non-digestive techniques were studied. The digestive technique is the analysis of phosphate in the phospholipid fractions after column chromatography. The non-digestive techniques are based on the extraction of a phospholipid-chromogenic reagent association complex from the HPLC solvent into a suitable immiscible organic solvent. The first chromogenic reagent used was Vaskovsky and Kostetsky spray reagent (a mixture of Mo^{V} and Mo^{VI} in acidic conditions). Using this reagent the quantification of PE, PS, PI, PC, DPG and Spm in HPLC eluent was possible. A second chromogenic reagent was developed by reduction of molybdate by 1-aminonaphthol sulfonic acid. Quantification of PE, Spm and PC in the HPLC eluent after column separation was achieved with this reagent. Both the above reagents were used without a heating step for color development, because the phospholipids were found to complex with compounds in the molybdenum blue form. Molybdenum blue reagents can be added directly to the sample allowing extraction of the molybdenum blue-phospholipid complex into a suitable immiscible organic solvent for quantification. The transfer of the phospholipid-chromogenic reagent complex is dependent on the class of phospholipid, the fatty acid side chains within each class, the acidity, the temperature and the extracting solvent used. Automation using these reagents was attempted and shown to be promising but further development is necessary.

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LIST OF ABBREVIATIONS

AM – ammonium molybdate

ANSA – aminonaphthol sulphonic acid

BPC – bonded phase chromatography

Chol – cholesterol

Chol E – cholesterol esters

DG – diglyceride

DPG – diphosphatidyl glycerol

DOPE – dioleoyl phosphatidyl ethanolamine

DOPC – dioleoyl phosphatidyl choline

DPPC – dipalmitoyl phosphatidyl choline

DPPE – dipalmitoyl phosphatidyl ethanolamine

DSPC – distearoyl phosphatidyl choline

DSPE – distearoyl phosphatidyl ethanolamine

FFA – free fatty acids

FID – transport flame ionization detector

HETP – height equivalent theoretical plate

HPLC – high performance liquid chromatography

LPC – lysophosphatidyl choline

LPE – lysophosphatidyl ethanolamine

LSC – liquid-solid chromatography

MG – monoglyceride

MGP – monoglycerophosphate

PA – phosphatidic acid

PC – phosphatidyl choline

PG – phosphatidyl glyceride

PE – phosphatidyl ethanolamine

PI – phosphatidyl inositol

PL – polar lipids

PS – phosphatidyl serine

RI – refractive index

Spm – sphingomyelin

ST – sterol

TG – triglyceride

TLE – total lipid extract

TLC – thin layer chromatography

UV – ultraviolet

STATEMENT OF THE PROBLEM

Phospholipids are essential structural components of membranes and have other important biological functions. The analysis of phospholipids has helped answer many questions varying from the mechanism of aging in meat to the onset of a pathological condition in humans. Thin layer chromatography (TLC) has been the most widely used method for the analysis of phospholipids. This technique requires two-dimensional development, scraping and extraction to remove the lipid from the plate, and a subsequent inorganic phosphate analysis after acid digestion.

High performance liquid chromatography (HPLC) appears to have the potential to be an alternative to TLC methodology, if an appropriate method of detection is developed. The new ultraviolet (UV) detectors have made certain types of qualitative analysis possible, but quantitative measurements are difficult. Use of UV detectors is unreliable due to variability in extinction coefficients, caused by the presence of a variety of fatty acid side-chains within each phospholipid class. Acid digestion and inorganic phosphate analysis can be used for post column detection, but this is tedious and time-consuming, hence it draws away from the advantages of a rapid HPLC separation.

The objective of this research work is to investigate the separation of phospholipids by HPLC and examine the potential of non-digestive colorimetric techniques to quantify the phospholipid in the column eluent after separation.

CHAPTER I

INTRODUCTION

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Introduction

Chromatography in its many varied forms is one of the analytical chemist's most important tools. The discovery of chromatography has been credited to Michael Tswett (1) from his paper in 1903 describing the separation of plant pigments on chalk. In 1941, Martin and Synge (2) published theoretical and applied work on chromatography, which led to the development of paper chromatography in 1944 (3) and gas chromatography in 1952 (4). The rapid development of gas chromatography from 1952 to the late 1960's laid the ground work for the development of modern liquid chromatography. In 1967, Huber and Hulsman (5) were among the first to describe a liquid chromatography technique comparable in resolution and speed to gas chromatography. Snyder (6) and Kirkland (7) at the same time made major contributions to liquid chromatographic theory and technique leading to the development of the modern form referred to as HPLC.

The technique of HPLC uses high pressures and small bore columns containing microparticle packing material. Recently, HPLC is finding wide and varied applications in many areas of chemical and biochemical analysis. HPLC has the advantages over other forms of liquid chromatography of high resolution, shorter analysis times, less operator error, automatic operation, and high sensitivity. There have been a large number of reports published on the subject (8) and several new books covering all aspects of the technique (9–14) can be obtained.

Theory

Chromatography is a separation technique based on a simple partitioning between a moving phase (gas or liquid) and a stationary phase (liquid or solid). The objective of chromatography is to separate a number of compounds in a sample into bands by selective retention on a stationary phase. The retention of a component is defined by the term retention time (t_R), the time from injection to the peak maxima as obtained on a suitable recording device. The t_m is the time required for a component to travel through the column without being retained. The adjusted retention time (t_R') is calculated by subtracting t_m from t_R (see Figure 1 for the definition of chromatographic terms). The value of t_R is dependent on a component-specific equilibrium distribution between the stationary and mobile phases. Components which favor the mobile phase move faster through the column than those which favor the stationary phase.

The resolution, R, which is the degree of separation, is measured by the equation:

$$R = \frac{t_{R_2} - t_{R_1}}{\frac{w_2 + w_1}{2}} = \frac{2\Delta t}{w_2 + w_1}$$

where t_{R_2} and t_{R_1} are the retention times of the components measured at the peak maxima. Δt is the difference in these times. w_1 and w_2 are the widths of the bases of the peaks in units of time. For the best resolution, narrow peaks and good separation between peak maxima is required. The narrowness of the peak is dependent on the column efficiency. Separation is dependent on column selectivity.

Column efficiency is the ability of the column to minimize peak spreading as a component moves through the column. A column requires high efficiency if a large number of components with close retention times are to be separated. Column efficiency for each component can be approximated by N, the number of theoretical plates, using

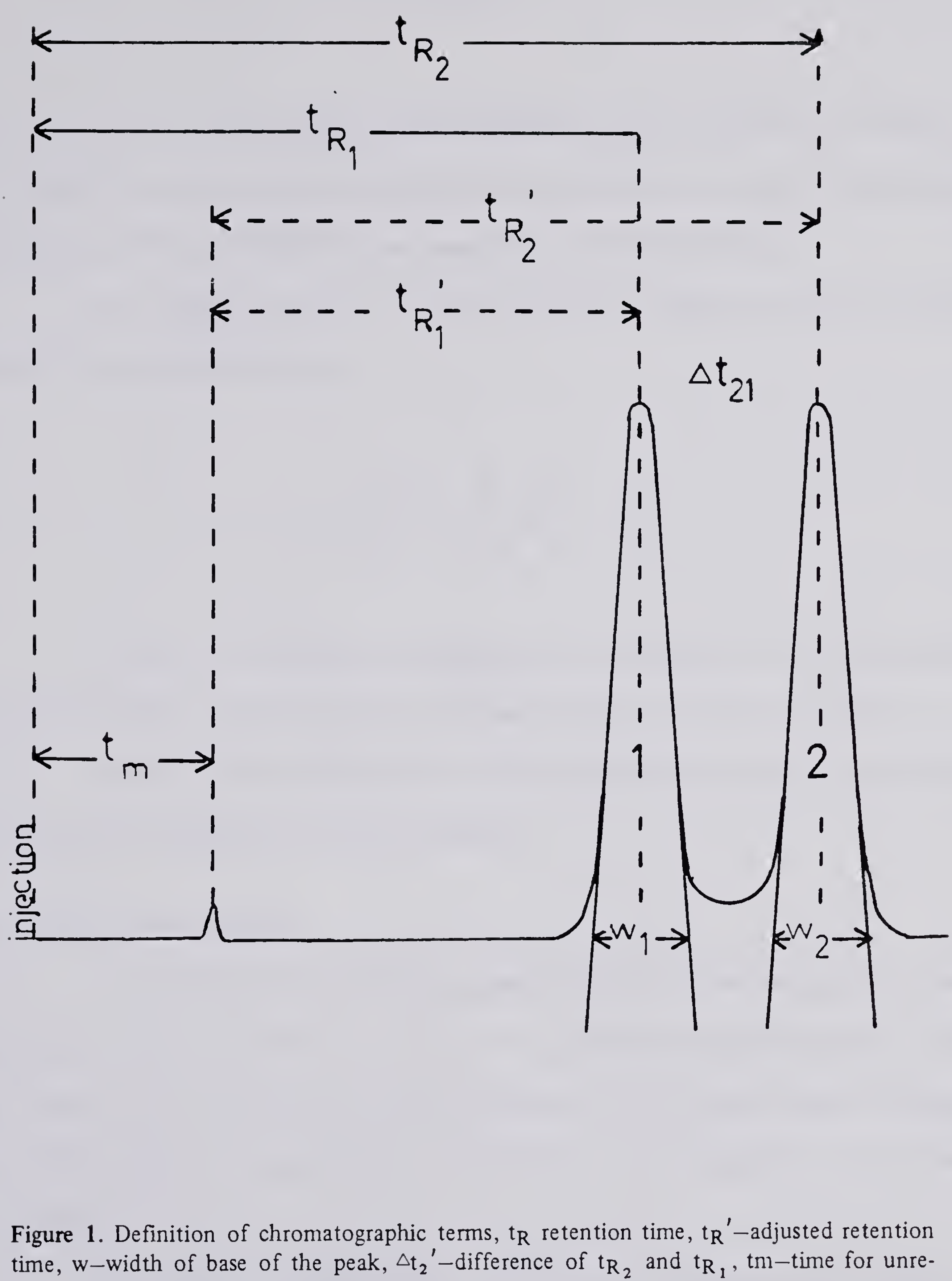


Figure 1. Definition of chromatographic terms, t_R retention time, t_R'—adjusted retention time, w—width of base of the peak, $\Delta t_2'$—difference of t_{R_2} and t_{R_1}, tm—time for unretained components (solvent front).

the equation:

$$N = \frac{16t_R^2}{w}$$

where w is peak width. The height equivalent theoretical plate (HETP) is N divided by column length. This is the preferred measure of column efficiency because it is independent of column length. The use of HETP allows comparison of different columns.

The column selectivity, a, is the ability of the column to separate two components and is given as the equation:

$$a = \frac{t_{R_2} - t_m}{t_{R_1} - t_m} = \frac{t_{R'_2}}{t_{R'_1}} = \frac{k_2}{k_1}$$

k_1 and k_2 are distribution coefficients of components 1 and 2 respectively. In the above equation a should be less than 1 for good separation of two components.

Snyder (15) has shown how theoretical calculations can aid in selecting the best experimental conditions for an HPLC separation.

Types of Liquid Chromatography

An understanding of the basic types of liquid chromatography is important in choosing the column and solvent system for the separation of specific classes of compounds. There are four primary separation modes used in HPLC: liquid-solid chromatography (LSC), partition chromatography, ion-exchange chromatography, and exclusion chromatography.

Liquid-solid chromatography, or adsorption chromatography, is based on a solute partitioning between a mobile phase and a solid stationary phase. The stationary phase used is usually silica gel or alumina. The mobile phase may be a non-polar solvent such

as hexane or chloroform. The solute molecules in the mobile phase compete with the solvent molecules for sites on the adsorbent. The technique is generally applied to solutes which are non-ionic and soluble in organic solvents. Saunders (16) has recently reviewed the practical aspects of this technique.

Partition or liquid-liquid chromatography involves a liquid mobile phase and a liquid stationary phase which is dispersed on an inert support material or chemically bound (bonded phase chromatography) to the support. The sample partitions between the mobile phase and the stationary phase causing differential migration and separation. The two liquid phases must be miscible.

In bonded phase chromatography (BPC), if the stationary phase is more polar than the mobile phase it is referred to as "normal phase"; if the mobile phase is more polar than the stationary phase it is referred to as "reverse phase".

Ion exchange chromatography works on the principle of substitution of one ionic species for another. The mobile phase is saturated with an anion (X^-) when an anion exchanger (R^+) packing is used. A negatively charged solute Y^- competes with X^- for association with R^+ on the stationary phase. The charges are the opposite if a cation exchanger is used. The separation is dependent on the degree of association of the solute with the ion exchange packing.

The fourth type of chromatography is gel permeation or exclusion chromatography which separates molecules on the basis of molecular size. The stationary phase consists of an inert porous-surfaced gel. In HPLC usually a semi-rigid packing material of cross-linked polystyrene is used over a soft gel such as Sephadex which is highly compressible and easily damaged by high flow rates.

Separation takes place because small molecules travel through a network in the beads and the larger molecules elute more rapidly because they are not retained in the network. A molecular weight difference of at least 10% is required for separation to take place.

Understanding the properties of column packing and their mode of separation has great practical application in selecting chromatographic conditions. Johnson and Stevenson (9) discuss the practical aspects of each mode of chromatography in detail.

Apparatus of HPLC

An understanding of HPLC apparatus may help one choose the equipment needed for a specific separation. A basic system consists of a liquid reservoir, a pump, a sample injector, a column, a detector and a recorder. Some systems have an oven for temperature control of the column and gradient elution devices are also available. HPLC equipment has been reviewed by a number of authors (9, 17, 18).

There are three major types of pumps: syringe, reciprocating, and constant pressure. Most of these pumps are capable of operating at pressures exceeding 1,000 psi. The newer HPLC pumps can operate to pressures of 5,000 psi, but conditions requiring these pressures are rarely required for separations.

For some separations which have compounds varying greatly in polarity (such as phospholipids), gradient elution conditions may be required. A gradient elution system usually has two pumps which are programmed to mix two miscible solvents in varying amounts to create the gradient.

After the pump the next piece of apparatus in the HPLC system is the injector. The most common injector used in commercial HPLC systems is the automatic loop injector, though septum injectors and stop flow injectors are also used. In the automatic loop injector, the sample is injected into a loop of tubing of a specific volume; the loop is then connected into the stream flow injecting the measured amount of sample. The injector is basically a six-way valve.

The column is the most important part of the chromatography system and is found just after the injector in the flow stream of the HPLC. In the late 1960's and early 1970's, most HPLC columns were packed with porous layered beads (30–40 μ), but more recently microparticles (5 and 10 μ) with higher column efficiency, sample capacity, and

speed of analysis have been used. Packing microparticle columns is difficult so most workers purchase prepacked columns.

Most prepacked analytical columns are 25 or 30 cm in length and 4.0 to 4.6 mm in diameter, but some are as small as 2.2 mm and some as large as 5 mm. There are relatively few types of stationary phases (adsorbent, bonded phase, ion exchange and exclusion) but within each type there may be as many as 25 different commercial varieties. Major (19) has recently reviewed HPLC packing and columns.

After separation on the column the solute must be quantified by some form of detector. The most common detector used in HPLC is the ultraviolet absorption detector because of its ease of operation and sensitivity. The absorption detectors are either fixed wavelength (usually 254 or 280 nm or both) or variable wavelength (usually between 190 and 600 nm). Refractive index monitors are not as common as the UV detectors. They measure changes in refractive index of a solution caused by the presence of a solute. They are considered a universal detector but are incompatible with gradient elution techniques.

Some of the less common detectors used for HPLC are fluorescence, polarographic, transport flame ionization, atomic absorption and mass spectrometric. The specific principles and applications of detectors have been reviewed by a few authors (17, 18).

For an HPLC user it is extremely important to recognize the rapid advancements in HPLC apparatus. Every year improvements in columns, pumping systems and detectors make new types of analyses possible.

THE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PHOSPHOLIPIDS

The rapid development of HPLC of lipids over the past few years has been due to technological advances in the areas of columns, detectors and pumps. The progress in liquid chromatography of lipids has been reviewed by a number of authors (20–24).

HPLC has been successfully applied to the separation and detection of neutral lipids (25–30) but phospholipids have required more specialized conditions, as they

differ greatly in polarity between classes and in fatty acid composition within a class. These two variables have led to a wide variety of different chromatographic and detection systems for phospholipid analysis.

The most common chromatographic systems used have been adsorption on silica gel (31–41), or silicic acid (42–45). Some workers have designed systems using reverse phase (46), gel permeation (47), and anion exchange columns (48, 49).

The solvent system most commonly used is a combination of chloroform, methanol and water (31, 32, 42, 46, 49), but in studies using UV detectors, other solvent systems had to be employed in the separation (37–41). In some cases isocratic (non-gradient) conditions have been used (23, 46) but gradient elution is the more common technique if the separation of all polar classes is required (46).

The detection systems include transport flame ionization detector (FID) (32–35, 43, 44, 46, 49–53), refractive index monitor (47, 54, 55), fluorescence detector (56), direct ultraviolet (UV) monitoring (37–41), ultraviolet monitoring after derivatization (36), and fraction collection with subsequent chemical analysis (31, 42, 48, 57, 58). Most of these systems are subject to limitations.

Before the advent of modern commercial HPLC systems, chemical analysis of fractions was the most typical method of detection of phospholipids and is still necessary if a high degree of accuracy is required.

Fischer and Kabara (57) separated mouse liver total lipid extracts on Florisil using multibore columns. They found that the separation of lipid classes was facilitated when the column diameter was decreased. They used chemical analysis as a means of detection and were able to separate Chol E, TG, Chol, DG, MG, FFA, and PL using a stepwise elution gradient of mixtures of hexane, ether, and methanol.

Nelson (31) built a very sophisticated gradient elution system for the separation of whole rat brain extracts on a silica gel column at a flow rate of 1 ml/min, using a concave gradient of 100% chloroform to 100% methanol. The chemical analysis of frac-

tions showed the possible separation of PI, PE and PS, LPE, PC and Spm in 1,000 minutes.

Montet *et al.* (42) separated a total lipid extract of human bile using high pressure (20–30 kg/cm^2) on a silicic acid column with fraction collection and subsequent automated phosphate analysis. He showed the separation of PE and PS in one peak, PC, Spm and LPC in separate peaks using a stepwise gradient of chloroform, methanol and water mixtures.

Dittmer (58) separated PE, PG, DPG of an *E. coli* polar lipid extract on DEAE (diethylaminoethoxypropylated) Sephadex LH-20 column under atmospheric pressure. A gradient of ammonium formate in chloroform methanol was used and fractions were quantified by an automated phosphate analysis.

Lairon *et al.* (48) separated PC, MGP and PO_4^{3-} using an anion exchange resin under pressure in a two-step elution mode using ammonium formate-sodium tetraborate and HCl. The fractions collected were analyzed by an automated phosphate analysis.

The most widely used detector in lipid analysis until recently has been the transport flame ionization (FID) detector (20). Karmen *et al.* (50) first suggested that the FID be used for phospholipid analysis.

Stouffer and Oakes (32), using a moving chain transport FID, analyzed crude soybean lecithin on a silica gel H column. There were two major peaks detected using a chloroform, methanol, water solvent system pressurized with N_2 gas.

Worth and MacLeod (43), using a moving wire FID, detected the separation of Chol E, TG, FFA, DG, MG and PL on a silicic acid column. They applied the method to normal blood plasma and serum lipids. Later Worth (44) applied the method to serum lipid analysis of patients with nephrotic syndrome and diabetes. Alme and Sjovall (46), using a moving chain FID, separated microgram quantities of eight synthetic phosphatidyl cholines and four synthetic lysophosphatidyl cholines according to fatty acid chain length. Stolyhwo and Privett (51), using the moving wire FID, showed the separation of 28 lipid components of red blood cell extracts on a Corasil II column. A continuous gradient was employed, using

pentane, ethyl ether, chloroform and methanol, containing 8% ammonium hydroxide. They also showed separation of reference mixtures of polar and nonpolar lipids. Privett *et al.* (52) applied the same system to the analysis of lipid extracts of soybean at different maturation stages. Erdahl *et al.* (53) repeated this work using soybean lecithin.

Rainey (23) and Rainey *et al.* (33, 34) used "simplex optimization", which is a statistical design for finding the optimum response, to develop a system for the separation of phospholipids. A moving wire FID detector was used to monitor the separation of LPC, PE, PI, PS, PC, Spm and PA on a Corasil II column with a chloroform:methanol:ammonia (50.0:35.9:7.0 by vol.) solvent system. These workers showed only the separation of standards. Blood sample extracts were not separated successfully.

Kiuchi *et al.* (35) analyzed soybean lecithin on a Micropak SH-10 silica gel column using a moving wire FID. A gradient system starting with hexane:chloroform (9:1 by vol.) followed by an ethanol concentration gradient of 3% per minute separated the soybean lecithin into TG, DG, ST, FFA, MG and two peaks of PL.

Later Kiuchi *et al.* (49) focused specifically on phospholipids using moving wire FID. They separated synthetic PA, PG, PS, PC, PE and tristearin on an anion exchanger Bondapak NH_2 column with a pre-column packed with AX/Corasil. The solvent system consisted of 80% methanol:water (25:1 by vol.) and 20% chloroform.

The transport flame ionization detector has been widely used for lipid analysis (20). It has two disadvantages: (a) it has to be calibrated with synthetic lipids because it quantifies carbon number; (b) it is not presently commercially available because it has not found wide application outside of lipid analysis. Ultraviolet detection is a far more widely used detection system outside of the lipid area.

The refractive index (RI) monitor has been applied to analyze the HPLC eluates of phospholipid separations. Randau and Bayer (47) investigated egg lecithin on a gel permeation column using an RI monitor. Arvidson (54), using the RI monitor, also looked at egg yolk lecithin on a column of hydroxyalkoxypropyl Sephadex LH-20. Four

components were resolved in a methanol:water solvent system. Patel and Sparrow (55) also used the RI monitor for their large-scale purification of crude egg phospholipids.

Fluorescence detection was used by Asmus *et al.* (56) based on the shift of 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence when ANS interacts with phospholipids. The phospholipid separation took place on a Corasil II column eluted with 1% NaOH in methanol. This system was able to detect the separation of PC and DPG.

Before the availability of variable wavelength UV detectors for liquid chromatography, phospholipids were not detectable by fixed wavelength UV detectors (set at 254 nm or 280 nm). Jungalwala *et al.* (36) overcame the detection problem by derivatization of amine-containing phospholipids. They formed biphenylcarbonyl derivatives of PE, PS and LPE which absorb at 280 nm. They separated the derivatized phospholipids in two solvent systems on Micropak SI-10. Later McCluer and Jungalwala (37) used a gradient elution system and were able to separate PE, LPE and PS from a single injection. They were also able to show the effect of fatty acid chain length on the elution profile for three phosphatidyl ethanolamines: DSPE, DOPE and DPPE. DSPE and DPPE had a retention time of 5.3 minutes where DOPE had a retention time of 4.9 minutes.

In order to analyze the nonamine-containing phospholipids, Jungalwala *et al.* (38) and McCluer *et al.* (39) used far UV detection at 203–205 nm. A low-absorbing solvent system consisting of acetonitrile:methanol:water (65:21:14 by vol) was used on a Micropak SI-10 silica gel column to separate PC and Spm with UV detection at 203 nm. PE and PS appeared as a shoulder in the solvent front.

Using synthetic phosphatidyl cholines with varying fatty acid chains, McCluer *et al.* (39) found that UV absorption at 203 nm was a function of the degree of unsaturation. Therefore it is a requirement to determine the apparent extinction coefficients of source materials prior to quantification with UV. They applied the system to human amniotic fluid, human erythrocytes, rat brain and rat liver extracts.

McCluer and Jungalwala (37) developed a gradient elution solvent system

to move the PS and PE away from the solvent front. They used a Micropak SI-10 column with a 10 minute linear gradient of 15% to 35% methanol, water, ammonia in acetonitrile. Four peaks, PE, PS, PC and Spm were detected at 205 nm for a rat brain lipid extract.

Geurts Van Kessel *et al.* (40) showed almost complete polar lipid class separation using UV detection at 206 nm. A gradient elution system of n-hexane:2-propanol: water on a Li Chrosorb Si-60 10 μ silica gel column was used to separate Chol, PA, PE, LPE, PI, PS, Spm-PC, Spm and LPC of a total lipid extract of human erythrocyte membranes. Spm and PC were not resolved. They also showed the separation of synthetic dioleoyl standards of PA, PE, PG and PC using an isocratic solvent system of n-hexane:2-isopropanol:water. Phosphatidyl cholines of great variation in fatty acid chain could be separated using solvent systems with high water content.

Geurts Van Kessel *et al.* (40), like Jungalwala *et al.* (38), studied the absorbance behavior of synthetic phospholipids and concluded that due to the great variety of fatty acid constituents within a lipid class direct quantification was not possible without estimation of molar extinction coefficients for each source being studied. Geurts Van Kessel *et al.* (40) inferred that peroxidation of the double bond may cause a change in retention and an increase in molar extinction coefficient after observing a second peak next to DOPC when testing a reaction mixture of the reacylation of 1-glycero phosphocholine with oleoyl chloride.

Later Hax and Geurts Van Kessel (41) looked at this system for the separation of polar lipids from suboesophageal ganglia of the snail *Helix pomatia.* This extract was chromatographed in a two-step gradient of hexane:2-propanol:water, and showed separation of Chol, PA, PI, PE, PS, PC, and LPC on a Spherisorb SIOW column. Sphingomyelin was separated into two components on Li Chrosorb Si-60 5 μ silica gel column, one containing behenic and lignoceric fatty acids, and a second with palmitic fatty acids. This explained the contamination of the PC peak by Spm in the human erythrocyte membrane separations.

Recently three large-scale preparative systems have been developed for the separation of crude phospholipids plus a preliminary subfractionation by fatty acid composition within each lipid class. Fager *et al.* (45) separated 10 grams of crude phospholipids prepared from egg yolks on eight columns (1 m x .397 in) connected in series containing Biosil HA minus 325 mesh silicic acid. A stepwise gradient of chloroform with increasing methanol from 10% to 50% was used. This system was capable of separating PC, PI, PE and LPE, plus a preliminary subfractionation of each lipid class.

Patel and Sparrow (55) developed a large-scale purification system using the Waters Assoc. Preparative LC-500 Liquid Chromatograph and a Pre Pack 500 cartridge containing silica gel to purify crude egg phospholipids with a chloroform, methanol, water solvent system. Neutral lipids, then phosphatidyl ethanolamine and finally phosphatidyl choline were eluted from the column. The system was monitored with an RI monitor and TLC.

A third preparative system was developed by Radin (59) for the purification of lecithin on a Lobar silica gel column using a two-step gradient of hexane, isopropanol, water. This technique only purified the lecithin and did not separate the impurities into distinct phospholipid fractions.

The application of HPLC for the analysis of phospholipids has paralleled the development of HPLC instrumentation. Since the availability of the variable wavelength detector which detects phospholipid in the far UV, there have been rapid advances especially in the area of separation, though quantitative detection is still a serious problem. In this thesis I have used the above separation systems, but attempts have been made to find a simple method to overcome the serious problems in quantitative detection.

DIGESTIVE COLORIMETRIC TECHNIQUES FOR THE ANALYSIS OF PHOSPHOLIPIDS

The most commonly used method for the quantification of phospholipids either after extraction or after separation by thin layer chromatography or column chroma-

tography is digestion to liberate inorganic phosphate. The phosphate is determined colorimetrically, through the formation of molybdophosphoric acid in an acid medium with subsequent reduction to heteropoly molybdenum blue. Garfield (60) lists the three most cited papers on this type of method as Fiske and Subbarow (61), Bartlett (62), and Chen, Toribara, and Warner (63). Baginski *et al.* (64) and Lindberg and Ernsten (65) have reviewed the methods of organic phosphorus analysis using digestion. Baginski *et al.* (66) have also reviewed phosphate methodologies including direct measurement of inorganic phosphate.

The literature on methodology of organic and inorganic phosphorus analysis is extensive. There are a large number of methods for digestion of phospholipids found in the literature (64). The most commonly used method for digestion is heating with sulfuric acid and hydrogen peroxide as originally suggested by Baumann (67) and Briggs (68). Perchloric acid has been extensively used (69–71) but the risk of explosion by peroxide formation has been noted (72). A few examples of other digestion systems that have been used are: sulfuric-perchloric (73), sulfuric-nitric (63), sulfuric-selenious (74), and sulfuric-nitric-H_2O_2 (75).

After phospholipid digestion, inorganic phosphate is usually analyzed by reacting molybdate with phosphate to form molybdophosphoric acid which is then reduced to heteropolymolybdenum blue (64). The chromophore is measured at 700 nm to 840 nm. The unreduced form of molybdophosphoric acid (76) and vanadomolybdophosphoric acid (77) have also been measured. In some cases where no reduction is employed, a basic dye has been reacted with molybdophosphoric acid to form a strongly absorbing chromatophore (69–71, 78).

There have been a large number of reducing agents employed under varying conditions (64). The most frequently used reducing agent is 1-amino-2-naphthol-4-sulfonic acid, first suggested by Fiske and Subbarow (61). Other important reducing agents used in phospholipid analysis include ascorbic acid (79), hydrazine sulfate (80), ferrous sulfate (81), tin chloride (82), and diaminophenol (83). The pH is critical, the addition of acid or base

may be required for optimum reaction conditions in a digested sample (64).

Usually the phosphate is reacted with molybdate and then reduced but Vaskovsky *et al.* (69) have outlined a method where molybdate is reduced prior to reaction with phosphate. The solution is stabilized with concentrated sulfuric acid.

Many workers have used the interaction of basic dyes with molybdophosphate to form strongly absorbing chromophores, which greatly increase the sensitivity of phosphate assays. Some of the dyes used are quinaldine red (84), safranine (85), crystal violet (86) and malachite green (70, 71, 78, 87). Malachite green was first used by Itaya and Ui (78) and has found the greatest use among lipid chemists because of its sensitivity (70, 71, 87). Usually in these methods the malachite green and molybdate are added as one reagent but Petitou *et al.* (87) add ammonium molybdate and then malachite green. The malachite methods have increased sensitivity of phosphate analysis and are very useful in micro TLC (71). They also may be useful in the quantification of phospholipids after HPLC separation.

The basic chemistry of phospholipid analysis really has not changed very much over the years; most improvements have involved changes which increase sensitivity and decrease manipulation time needed to complete the analysis. It is one of the objects of this thesis to investigate non-digestive techniques which may replace the tedious digestive techniques requiring the use of strong acids and heating.

NON-DIGESTIVE COLORIMETRIC TECHNIQUES FOR THE ANALYSIS OF PHOSPHOLIPIDS

A number of non-digestive colorimetric techniques for the analysis of phospholipids have been developed in the last decade (88–92). These are generally based on the association of phospholipids with molybdate compounds. This association complex is usually reduced and the absorbance of molybdate blue is read in the 700–800 nm range. The association complex is insoluble in aqueous solutions and is usually extracted by lipophilic

organic solvents (88, 89, 91, 92) or resolubilized with a surfactant and a miscible solvent (90) prior to quantification. The extraction method seems to be the most popular because there is no interference from molybdate-phosphate complexes.

In order to have a better understanding of the chemistry behind non-digestive techniques of phospholipid analysis, a discussion of phospholipid reagents is necessary. These reagents have been used in both TLC spray reagents (95), and in non-digestive colorimetric techniques (89, 91). In both cases, there are two types of complexes formed: (a) molybdate-phospholipid and (b) phosphomolybdate-phospholipid.

The mechanism by which the molybdate complexes with phospholipid is unknown. It is possibly significant, however, that Mo^{V} oxidation state is present in all the molybdate phospholipid reagents whether they are used as TLC spray reagents (93–97) or as reagents in extraction techniques (88, 89, 91, 92). The Mo^{V} is probably critical to detection of the complex since it readily forms molybdenum blue when associated with phospholipids under acid conditions. The Mo^{V} state may be necessary for color development but it has been shown not to be necessary for association since unreduced molybdate can be extracted as a molybdate-phospholipid complex (88).

One of the most widely used phospholipid spray reagents is that of Dittmer and Lester (94) which is based on the Zinzadze reagent (98). It contains MoO_3 which is reduced to Mo^{V} state by powdered molybdenum and is then remixed with equal volumes of unreduced MoO_3 (Mo^{VI} state). The acidity is kept very high to maintain a stable solution.

The Vaskovsky and Kostetsky (95) spray reagent was used directly by Raheja *et al.* (89) and Sandhu (91) in their extraction methods. The reagent is essentially that of Lucena-Conde and Prat (99) developed for phosphorus analysis. Lucena-Conde and Prat (99) found maximum sensitivity at a ratio of 3:2 Mo^{VI} to Mo^{V}. This ratio is obtained by reducing ammonium molybdate with metallic mercury in HCl solution to the Mo^{V} state, then removing the residual mercury by filtration and adding unreduced ammonium molyb-

date to give a final ratio of 3:2 of Mo^{VI} to Mo^{V}. The stability of the solution is achieved by maintaining high acidity with 20 N H_2SO_4 and 1.3 N HCl.

Goswani and Frey (96) produced a spray reagent similar to that of Vaskovsky and Kostetsky (95) except metallic copper was used for reduction and no Mo^{VI} was added after the reduction. The final working solution had a much lower acidity (4 N HCl) than the Vaskovsky and Kostetsky spray reagent (95) (20 N H_2SO_4 1.3 N HCl).

Vaskovsky and Svetashev (93) re-evaluated their earlier spray reagent (95) and chose to use sodium molybdate instead of ammonium molybdate because of the higher solubility of the sodium salt. They found that they could reduce Mo^{VI} to Mo^{V} by heating 5 minutes at 100° C in 2 N HCl in the presence of hydrazine hydrochloride. The reduced molybdate solution was stable for several months without high acidity (approx. 0.4 N HCl).

Vaskovsky *et al.* (69) studied this reagent in detail for use as phospholipid spray reagent and in the analysis of phospholipid phosphorus following perchloric acid digestion of the lipid.

Kundu *et al.* (97) produced a similar spray reagent to the Vaskovsky and Kostetsky reagent (95). Ammonium molybdate was reduced with HI by heating in the presence of HCl. They crystallized the reduced compound, ammonium pentachlorooxomolybdate $[Mo^{V}OCl_5]^{2-}$, and redissolved it in 7–9 N H_2SO_4 for use as a spray reagent. They found it necessary to have a very high acid content to prevent the production of a blue background on the TLC plate.

Table I outlines the properties of these five spray reagents. They were shown to be specific for only phospholipids and gave a negative response to other neutral lipids, inorganic phosphate and various phosphorus compounds.

The color of each reagent is dependent on the acidity of the final working solution as shown by Table I. A solution containing 6–8 N HCl contains $[Mo^{V}OCl_5]^{-2}$ which is emerald green (97, 99). A solution containing 2–6 N HCl with $Mo^{V}O^{+3}$ present is reddish brown (97, 99). If the hydrochloric acid concentration is below 0.3 N molyb-

Table I

Composition of Molybdate Phospholipid Spray Reagents

Authors	Molybdenum Compound	Reducing Agent	Reducing Conditions	Mo^{VI} Readdition	Final Acidity	State	Specificity		Color
							Positive	Negative	
Dittmer & Lester (94)	molybdenum trioxide	powdered molybdenum	25 N H_2SO_4 100°C 15 min.	+	12.5 N H_2SO_4	Mo^{VI}:Mo^{V} 1:1	PC,PE,PI,PS, DPG,Spm	FA,TG,Chol, Sphingosine, Cerebroside	green yellow
Vaskovsky & Kostetsky (95)	ammonium molybdate	metallic mercury	4 N NCl Shaking 30 min.	+	1.3 N HCl 20 N H_2SO_4	Mo^{VI}:Mo^{V} 3:2	PC,PE,Spm, PE,DPG	FA,Chol, glycerol phosphates, fructose 6-P, AMP	green yellow
Goswani & Frey (96)	ammonium molybdate	metallic copper	12.5 N H_2SO_4 2 hr at 23°C	-	4 N H_2SO_4	Mo^{V}	PC,PS,PE, Spm	FA,Chol,TG, fructose 6-P, glycerol phosphates, Sphingosine, Cerebroside	dark brown
Vaskovsky & Svetashev (93, 69)	sodium molybdate	hydrazine hydrochloride	2 N HCl at 100°C for 5 min.	-	2 N H Cl	Mo^{V}	PC other PL see (95)	Assumed same as (95)	not stated
Kundu *et al.* (97)	ammonium molybdate	hydroiodic acid	conc. HCl with heating	-	7–9 N H_2SO_4	Mo^{V}	PC,LPC,PE, PI,PS,PA	sulfatide, Chol,FA, glycosphinolipids, inorganic phosphate	green yellow

denum blue is formed (93). This process is reversible by the addition of acid (94) with transitions to first reddish brown and then with increasing acid, to emerald green.

The type of acid and the acidity greatly affect sensitivity. Acidification with H_2SO_4 is desired over HCl because the chloride ion complexes with $[Mo^VO]^{3+}$ to form $[Mo^VOCl_5]^{2-}$ thus preventing color complex formation (97, 99, 69).

The acidity and reducing agent affects the extent and speed of reduction of Mo^{VI} to Mo^V which in turn affects the degree of coloration on the plate. The information regarding molybdate spray reagents can be applied in order to find a low acid and highly sensitive color reagent acceptable in extraction techniques, as these reagents are specific for phospholipids.

Another class of spray reagents uses phosphomolybdic acid instead of molybdate. These reagents are less specific, but are highly sensitive for choline containing phospholipids. Worth and Wright (92) used phosphomolybdic acid for the color reagent in their extraction technique. It was first used by Levine and Chargaff (100) as a spray reagent to detect choline head groups cleaved from phospholipids after reduction with stannous chloride.

Later Schneider (101) found it was not necessary to cleave the fatty acids from the phospholipid in order to attain identification with the spray reagent. He detected the phospholipids on TLC plates by using the phosphomolybdic acid with subsequent reduction with stannous chloride. His reagent was found to be sensitive for choline-containing phospholipids (Spm, PC) but it was nonspecific and also gave low response for cholesterol, PA, PE, PS, and sphingosine.

Phosphomolybdic acid reagents are useful in detection of Spm and PC in extraction techniques as shown by Worth and Wright (92). The Mo^V and the phosphomolybdate types of reagent discussed above are not the same. Schneider (101) stated that the plates are permanently stained with phosphomolybdic acid, where Mo^V compounds, when sprayed on TLC plates, start fading after 24 hours (97). This may give a clue as to the

type of complex formed by each of the two reagents.

The extraction phenomenon of molybdate-phospholipid complexes was first shown by Galanos (88). He showed that reduced and unreduced molybdate-phospholipid complexes were extractable under mildly acidic conditions into ethyl acetate and chloroform:ethanol (4:1 by vol.). When chloroform:ethanol was used, the extraction was selective for molybdate-phospholipid complexes whereas water soluble inorganic molybdate-phosphate complexes stayed in the aqueous layer. The assay was not affected by the presence of neutral lipids.

The phospholipids after complexing with molybdate gave 10% of the absorbance for an equivalent amount of phosphorus which would be formed during acid digestion of that phospholipid.

The molybdate could only be transferred in the presence of the phospholipid into chloroform:ethanol (4:1 by vol.) and there was a 5:1 ratio of molybdate to phosphorus for an isolated complex. Molybdate reacted with Spm, PC, PE and PI but no standard curve was given, possibly due to turbidity problems.

This extraction method indicates that an association complex, possibly analogous to those found by Vaskovsky and Svetashev (93) in their studies with TLC spray reagents, exists. The only essential difference in the two techniques is in the reducing agent used.

The most acceptable method of non-digestive, quantitative estimates of phospholipids was developed by Raheja *et al.* (89) using the Vaskovsky and Kostetsky spray reagent (95). His method had higher sensitivity than that of Galanos (88); probably because Raheja *et al.* (89) decreased the aqueous layer to a minimum (approximately 1:55 aqueous: organic). The aqueous layer was also maintained at high acid concentration, forcing lipids into the organic layer of chloroform.

The only problem with this method is that it assumed universality for all phospholipids, and employed only one standard curve. As stated by Van Gent and Roseleur

(90) and Worth and Wright (92), this is probably not the case; in their systems they found response to be dependent on the type of lipid.

Raheja *et al.* (89) showed that inorganic phosphate and neutral lipids did not interfere with the analysis. Sandhu (91) copied the Raheja *et al.* (89) method but instead of using chloroform he extracted with nonane which had two advantages. First, it produced less turbidity and second, unlike chloroform, it formed the top layer rather than the bottom layer. Even though it was easier to use, it was much less sensitive than the Raheja *et al.* (89) method possibly because chloroform would be expected to be a better lipid solvent than nonane. An examination of Sandhu's (91) standard curve (Figure 2) indicates that it is possible that a significant amount of the lipid was staying in the chloroform layer. Another drawback of Sandhu's (91) method is that lecithin was the only PL tested.

Worth and Wright (92) based their extraction method on the Levine and Chargaff (100) spray reagent using phosphomolybdic acid as the color reagent. They did a very detailed study isolating a dipalmitoyl phosphatidyl choline-phosphomolybdic acid complex, determining its molecular weight (4300) and carrying out an elemental analysis to find a ratio of 3:1 DPPC to phosphomolybdic acid. They also tested a large number of compounds to find the portion of the lipid molecule that the phosphomolybdate interacted with and postulated it to be the quaternary nitrogen. This is understandable because as shown by Levine and Chargaff (100) and Schneider (101), the reaction had high reactivity with choline containing compounds.

Schneider (101) showed that on TLC plates the reagent is nonspecific. This correlates well with Worth and Wright's (92) extraction data. The method is useful for the determination of PC/Spm ratios from amniotic fluid (102) because of its high reactivity with these compounds, but it is of less value for the analysis of PE, PS and PI which have low responses.

The transfer of both reduced and unreduced phosphomolybdic acid by

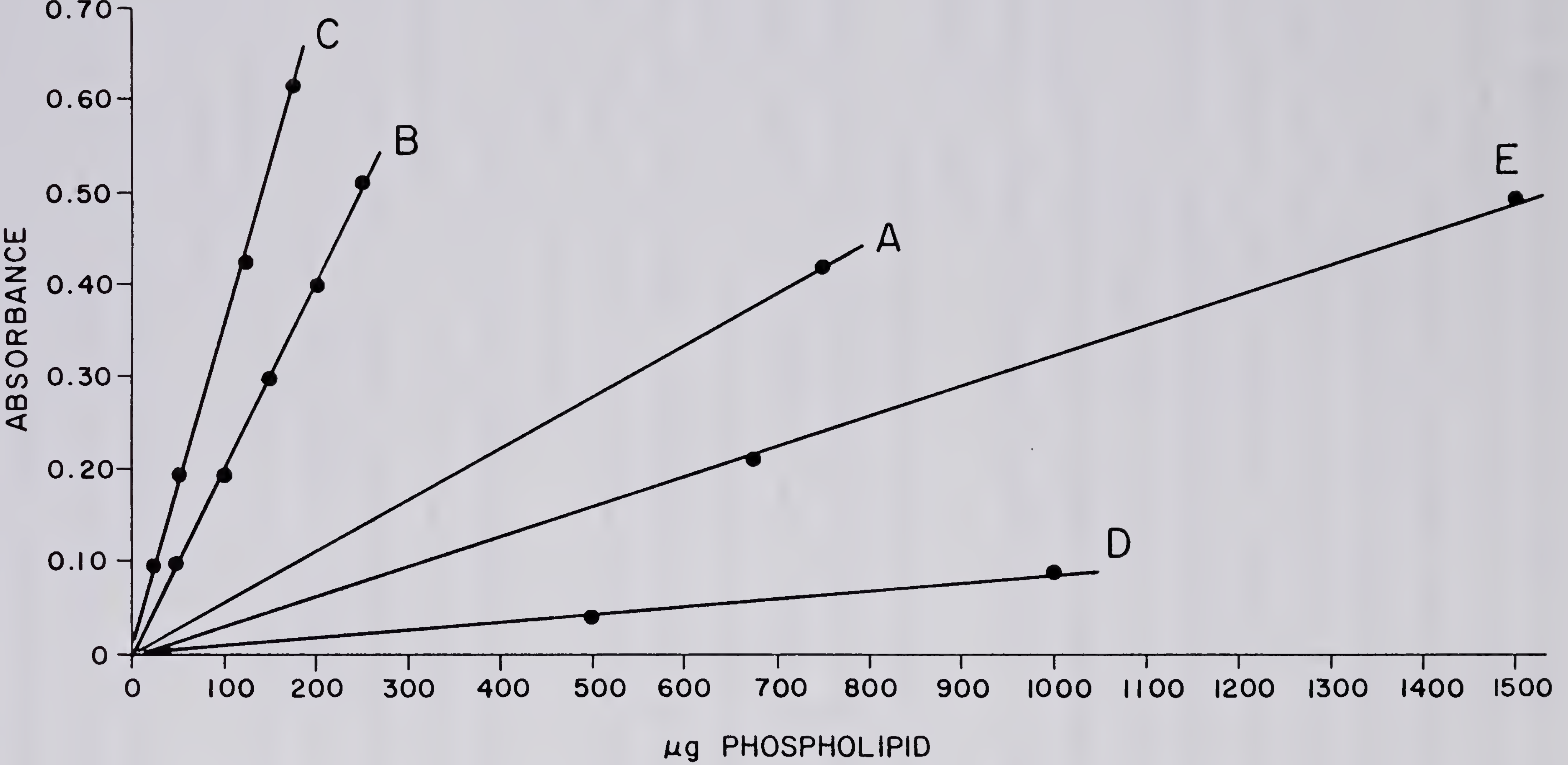


Figure 2. Standard curves for non-digestive colorimetric techniques. A–Galanos (88) technique–PL at 820 nm; B–Raheja *et al.* (89) technique–PC at 710 nm; C–Van Gent and Roseleur (90) technique–PC at 780 nm; D–Sandhu (91)–technique–PC at 710 nm; E–Worth and Wright (92) technique–PC at 690 nm.

lecithin was shown, but the reduced form of the complex was easier to detect. The sensitivity of even the reduced form is low as seen in Figure 2, as compared to Raheja *et al.* (89).

Worth and Wright (92) compared their method to Raheja *et al.* (89) and Sandhu (91) in a letter to the editor (103). They observed that the Sandhu and Raheja *et al.* reagents gave the same spectrum as molybdenum blue, but inferred that the phosphorus of the lipid was not the only reactive site on the lipid. They re-emphasized that the quaternary nitrogen, as shown in their method, was the probable site of interaction.

The reactivity and specificity as shown by the spray reagent data (discussed earlier) is completely different for molybdate complexes and phosphomolybdate complexes, but corresponds to the data of the analogous extraction techniques. The extraction methods are summarized in Table II.

An overlooked non-digestive colorimetric technique for the estimation of phospholipids is that of Van Gent and Roseleur (90). Instead of extracting the molybdate-phospholipid complex, they resolubilized it with a surfactant and butanol:dimethyl sulfoxide (1:1 by vol.). The color reagent used consisted of reduced ammonium molybdate (reducing agent hydrazine sulfate), vanadate-molybdate reagent (Merck), and a detergent solution (5% Tween). The lipid was mixed with the reagent and resolubilized with butanol: dimethyl sulfoxide (1:1 by vol.) after heating.

The method was able to detect phospholipids in the range of 25 to 200 μg giving a response approximately equivalent to the amount of phosphorus from phospholipid digestion. A standard curve for each lipid was necessary for PC, Spm, PE and LPC.

The color reagent is in a relatively low acid solution (3N H_2SO_4) and possesses an ochre color possibly due to Mo^VO^{+3}. When the reagent reacts with the phospholipid it forms molybdenum blue.

There are two non-destructive techniques not based on a molybdate reagent (104, 105). The first is that of Schiefer and Neuhoff (104) which complexes rhodamine 6G with phospholipids and measures the fluorescing phospholipid-rhodamine complex. They

Table II

Summary of Extraction Methods for theAnalysis of Phospholipids

Author	Molybdate Compound	Reducing Agent	Heating	Acidity	Extraction Solvent and Volume	λ max (nm)	Analysis Range (μg PL)	Specificity	
								Positive	Negative
Galanos (88)	Ammonium molybdate	1–2, 4 amino-naphthol sulfonate	10 min. 100° C	1.0 N $HClO_4$	5.0 ml $CH_3Cl/EtOH$ 4:1	820	-750 μg	PE,PI,PC, Spm	triolean, cholesterol, cerebroside
Raheja *et al.* (89)	Ammonium molybdate	metallic mercury	1–1.5 min 100° C	1.3 N HCl 20 N H_2SO_4	5.0 ml CH_3Cl	710	25–250 μg	PC,LPC,PE, PA, Spm	neutral lipid fraction
Sandhu (90) 1976	Ammonium molybdate	metallic mercury	1–1.5 min 100° C	1.3 N HCl 20 N H_2SO_4	2 ml nonane	710	1000-4000 μg	PC	Assumed same as (89)
Worth & Wright (91)	molybdo-phosphoric acid	metol (p-methyl-aminophenol) sodium bisulfate	no heat	no acid	3 ml $CH_3Cl/MCOH$ 2:1	690	-1400 μg	lipophilic compounds with quaternary ammonium group	Refer to paper (91)

found the reaction to be specific for phospholipids and neutral lipids did not interfere.

A second non-destructive method is that of Wotton (105). In his method the phospholipids are extracted into aqueous ethanol and then combined with ferric chloride. The ferric chloride accepts electrons from the hydroxyl ion and the ethoxide ion. The phospholipid replaces the anion in solution causing an increase in pH which is monitored by the indicator methyl orange.

The development of non-digestive techniques for the analysis of phospholipids has been slow due to a lack of understanding of the underlying mechanisms. Phospholipid spray reagent data in combination with extraction data may help resolve this problem.

The use of reduced molybdate Mo^V for complexing with phospholipid is specific and sensitive and would seem to be the reagent of choice. Vaskovsky and Svetashev's (93) TLC data show that high acid is not necessary for binding so if an analogous extraction system could be developed an automated system may be possible.

If extraction is used the nature of the solvent will be critical to the analysis because of different solubilities of different lipids and the effect of fatty acid chains. It is simple to see how sensitivity will drop when this is not considered (91).

The development of an improved non-digestive technique, coupled with a new separation technique such as HPLC and automation may lead to one-step phospholipid analysis after extraction of the phospholipids from natural sources.

CHAPTER II

MATERIALS AND METHODS

CHEMICALS

HPLC grade methanol, acetonitrile, hexane and isopropanol were used for HPLC separations. Reagent grade methanol and chloroform were distilled prior to use for lipid extractions. Mercury, ammonium molybdate, amino naphthol sulfonic acid, and ethyl ether were analytical grade. Benzene and toluene were spectroanalyzed grade. Perchloric, hydrochloric and sulfuric acids were reagent grade. 98% ethanol was used.

Phospholipids purchased are listed in Appendix I according to type and commercial source.

APPARATUS

A Spectra Physics Model 3500B, high performance liquid chromatograph was used for the development of the solvent system. The use of this instrument was provided by Mr. I. A. Simpson of Medical Laboratory Science Department at University of Alberta Hospital. This was a two-pump system using reciprocating piston-type pumps controlled by flow feedback electronic controls. The system had a solvent programmer so both isocratic and gradient conditions were possible. The detector was a Chromatronix Model 770 Spectrophotometric Detector capable of selection of wavelength between 200 and 450 nm when a deuterium lamp was used. The chromatograms were recorded on a Fisher Recordall series 5000 recorder. An Autolab minigrator was attached between the detector and the recorder to make quantitative peak area measurements possible. Figure 3 shows a schematic diagram of the experimental setup.

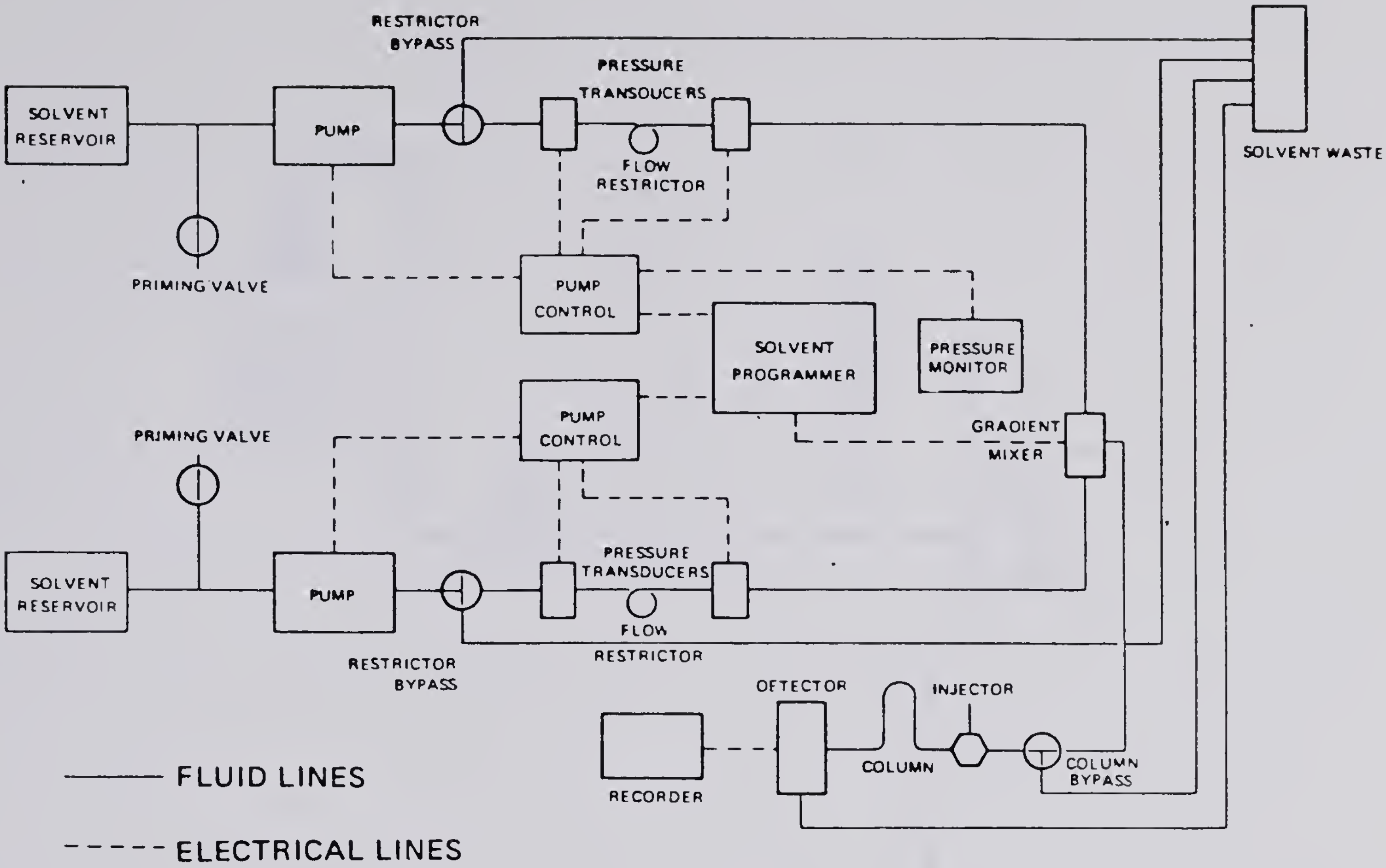


Figure 3. A schematic of Spectra Physics 3500B HPLC taken from the Spectra Physics 3500B Instrument Manual.

The use of a second HPLC system and Technicon Autoanalyzer II system was provided by Dr. J. A. Raleigh of Radiobiology Department at the Cross Cancer Institute, Edmonton, Alberta. The detector was on loan from Dr. S. Sarkar of Department of Food Science, University of Alberta. The system consisted of a Milton Roy minipump good to a pressure of 5,000 psi with a pulse dampener and a Rheodyne centre port injection valve with 20 μl loop. The detector used was a Tracor 970 variable wavelength detector good for wavelengths 190 to 650 nm. A Fisher Recordall Series 5000 recorder was attached to the UV detector for UV monitoring of the eluent. This sytem was coupled to a Technicon Autoanalyzer II system for simultaneous non-digestive automated colorimetric analysis. The HPLC system without the Technicon Autoanalyzer is shown in Figure 4.

A Vydac silica gel column (2.0 x 500 mm) provided by the Medical Laboratory Science Department was used with the Spectro Physics 3500B HPLC during the early

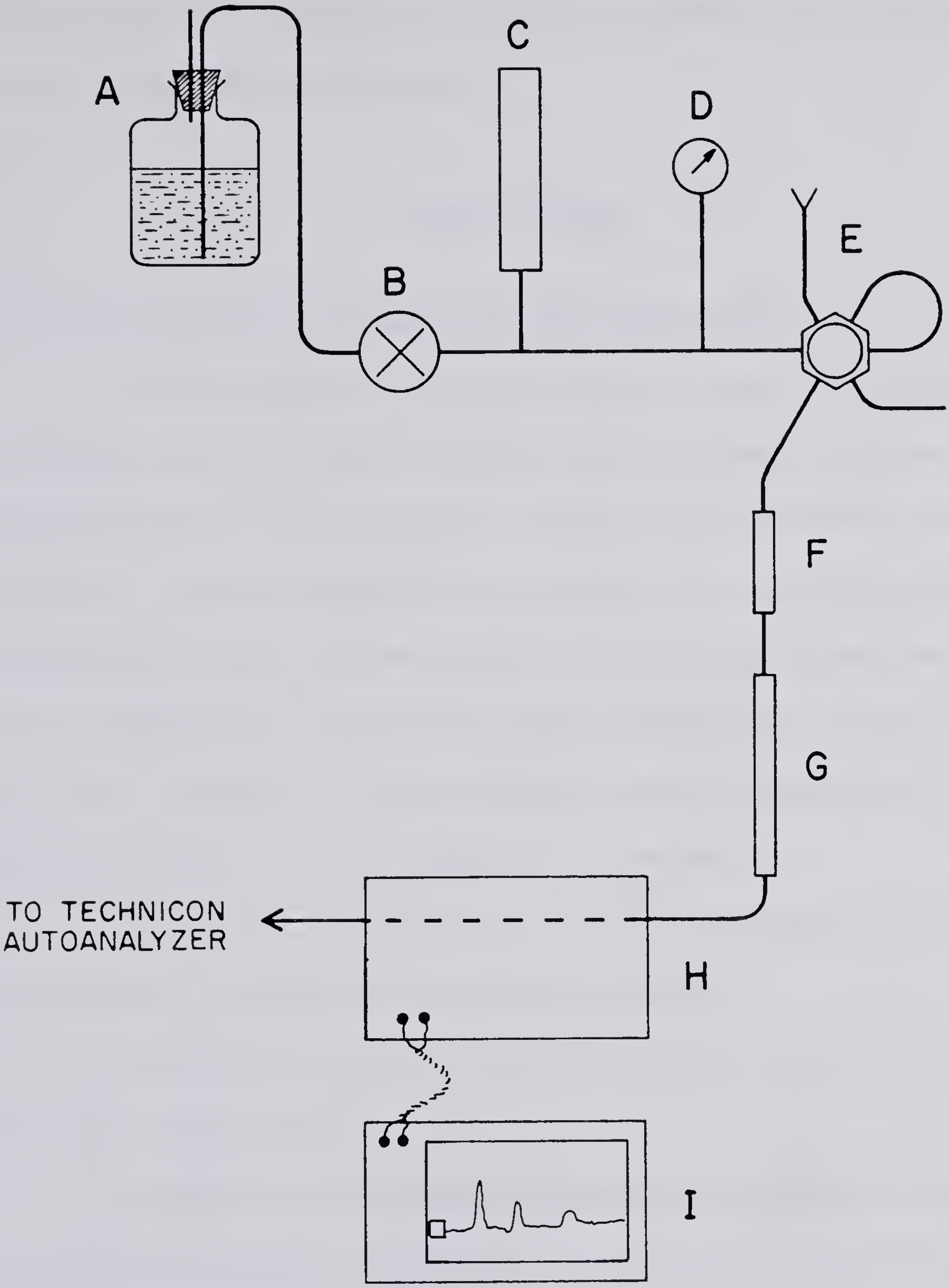


Figure 4. Milton Roy Mini Pump System with the Tracor 970 variable wavelength detector. A. solvent reservoir, B. pump, C. pulse dampener, D. pressure gauge, E. injector, F. Guard column, G. analytical column, H. variable wavelength detector, I. recorder.

solvent system development. The subsequent studies were done with a Whatman Partisol 10 μ silica gel column (4.6 x 500 mm). This system was equipped with a Whatman Guard Column (2 x 70 mm) packed with HC pellosil in both HPLC systems described earlier. The columns were periodically regenerated according to manufacturers' instructions. The pre-column was repacked at time of regeneration.

PROCEDURES

Standard Operating Conditions for the Spectra Physics 3500B HPLC

A methanol wash was first run through the column to remove contaminants from the solvent systems of other users, which might be present in the flow system. The chromatographic solvent was run through the column for 30 minutes prior to use. This also gave the detector a chance to stabilize. The detector was set at 0.01 Absorbance unit full scale at 205 nm and the flow rate was set from 1.0 to 3.0 ml per minute depending on the analysis. Two milliliters per minute was the most commonly used flow rate. The recorder was set at 10 mV full scale. The Autolab minigrator was set to attenuation between x2 and x16 signal (note at 0.01 A, x16 was considered 0.16 A full scale).

One milliliter of solvent was run through the injection port between sample applications to prevent contamination from previous injections.

In the Spectra Physics 3500B system, distilled and demineralized water, and HPLC quality solvents were used.

The solvent mixtures were prefiltered through a Millipore type LS 5.0 μm filter to remove any particulate contaminants. The pre-filtering also served as a degassing step, when slight agitation was used.

Standard Operating Conditions for the Milton Roy Mini Pump System with the Tracor Variable Wavelength Detector

The Milton Roy mini pump required priming prior to use. The flow was set at between 1.6 ml/min (18% full stroke) and 2.1 ml/min (25% full stroke). The sample was

injected through the centre port of the injector using a 20 μl loop.

The Tracor 970 variable wavelength detector was set at 205 nm. The solvent used in this system was not prefiltered or degassed prior to use. Distilled water was used in solvent system mixtures.

The above system was coupled to an Autoanalyzer II system for preliminary automation studies.

Lipid Extraction Techniques

Lipid extraction from beef muscle. The lipid extraction technique was based on Folch *et al.* (106) but the modifications suggested by Kramer and Hulan (107) and Bowyer and King (71) were used for a majority of phospholipids and analyzed by HPLC. The following technique was used for the preparation.

Weigh 2 grams tissue and freeze in liquid N_2. After freezing, the sample was crushed to a powder in a stainless steel mortar and pestle which had been cooled with dry ice. The powdered sample was then placed in 40 ml of chloroform:methanol (2:1 by vol.) in a Virtis Mixer and blended at medium speed for 3 minutes. It was then filtered through a prewashed No. 50 Whatman filter using a Millipore filter flask.

The filter and residue were placed in another 40 ml of chloroform:methanol (2:1 by vol.) and blending and filtration were repeated. The filter was rinsed with 5 ml of chloroform:methanol (2:1 by vol.). The extract was placed in a separatory funnel and the flask used in the filtration was rinsed with 5 ml chloroform. Then 17 ml of 0.9% NaCl was added to the extract and the mixture was shaken and allowed to stand at 4° C until separation of the phases was complete. The bottom layer was removed and stored at 4° C (fraction I). To the top layer 160 ml of chloroform:methanol:0.9% sodium chloride (86:14:1 by vol.) were added, shaken and stored at 4° C until separation of the phase was complete. The top layer was discarded and the bottom layer (fraction II) was added to fraction I. The combined fractions were rotoevaporated to dryness using a stream of N_2 when approaching dry-

ness. The residue was redissolved in 5 ml of chloroform:methanol:0.9% sodium chloride (86:14:1 by vol.), and stored at -20° C until further analysis. To prepare for HPLC injection the solvent was evaporated and the sample was redissolved in the appropriate solvent.

In some cases lipid extracts were separated (neutral from polar) using the column chromatography method as outlined by Hay *et al.* (108). It was later found that this was not necessary because the neutral lipids eluted at the solvent front.

Lipid extraction from chicken muscle. The lipid extraction technique used for chicken breast total lipid extracts was based on methods suggested by Kramer and Hulan (107) and Nelson (109).

Ten grams of tissue were removed from the muscle and placed in liquid nitrogen. The sample was crushed to a powder as in the previous method and placed in 250 ml chloroform:methanol (1:1 by vol.) which was cooled in an ice bath to 0° C and degassed with N_2. The sample was blended in a Waring Blender for 30 seconds. The extracts were immediately filtered through Whatman No. 50 (prewashed) filters using vacuum aspiration. Fifty milliliters of chloroform:methanol (1:1 by vol.) were used to wash the container and the filter. An additional 20 ml were added to the filter. One hundred and sixty milliliters of chloroform were used to wash the filter. The sample was then transferred to a one-liter round bottom flask and rotoevaporated maintaining a temperature below 30° C in the water bath. When approximately 50 ml remained in the flask, 200 ml of chloroform were added to remove water and rotoevaporation was continued. The sample was evaporated down to 10 ml and then filtered through a prewashed Whatman No. 50 filter to remove insoluble non-lipid contaminants. The sample then was evaporated under N_2 to a volume for loading on the column.

The column chromatography was carried out according to Nelson (109). Only fraction A was evaluated. It was rotoevaporated to a 5 ml volume and stored at -20° C until further analysis.

Phospholipid extraction from soya lecithin granules. Soya lecithin phospholipid extract was prepared for testing the non-digestive colorimetric system. Soya lecithin granules (Trophic Canada Ltd., Toronto) were extracted according to Folch *et al.* (106). Twenty-five grams of the granules were added to 500 ml of chloroform:methanol (2:1 by vol.) in a Waring Blender The sample was blended at 24° C for 3 minutes and filtered through a sintered glass filter. The extract was placed in a 500 ml separatory funnel and 100 ml of 0.74% KCl was added. The solvents were shaken and allowed to stand overnight at 4° C to separate. The bottom layer was removed and rotoevaporated to a volume of approximately 10 ml. A large silicic acid column was made as outlined by Hay *et al.* (108). Five grams of celite and 15 grams silicic acid were dried overnight at 110° C and packed in a column. The column was first washed with 600 ml of ethyl ether. This was fraction I containing neutral lipids. Then 500 ml of chloroform:methanol (1:1 by vol.) and subsequently 400 ml methanol were run through the column eluting fraction II containing the polar lipids. Fraction II was rotoevaporated to about 50 ml. The extract was dried with anhydrous sodium sulfate and filtered. The final solution after filtration contained approximately 45 mg/ml phospholipid as weighed by a Cahn-Gram Electrobalance.

Because of the nature of the thesis many methods of reagent formulation are outlined in the Results section.

The standard techniques which were not especially developed for this work are described in the Materials and Methods section.

CHAPTER III

RESULTS

DEVELOPMENT OF THE HPLC SOLVENT SYSTEM

The solvent system for the phospholipid separation was developed on a Spectra Physics 3500B high performance liquid chromatograph maintained in University Hospital, Edmonton, Alberta. The initial studies were undertaken with a Vydac silica gel 10 μ (500 x 2.0 mm) column as provided by the Medical Laboratory Science Department. The remainder of studies were carried out using a Whatman silica gel 10 μ (500 x 4.6 mm) column. Two principal solvent systems were evaluated: (a) the hexane:isopropanol:water system developed by Geurts Van Kessel *et al.* (40) and (b) the acetonitrile:methanol:water system developed by Jungalwala *et al.* (38). The second system, using the Whatman column, was found to give the most successful separations for the samples studied.

Preliminary Studies with the Vydac Silica Gel Column

The Acetonitrile:Methanol:Water Solvent System. An attempt was made to develop a solvent system for the separation of Spm and PC. Relatively low flow rates from 0.8 to 1.6 ml/min were used in these evaluations. The wavelength of the UV detector was set at 205 nm and ambient column temperature was used. Spm and PC were injected into the system as solutions in ethanol. Different ratios of acetonitrile:methanol:water were used to test the retention of PC and Spm.

The solvent ratios of acetonitrile:methanol:water (45:41:14 by vol.) and (65:21:14 by vol.) showed no retention of Spm or PC on the column. In acetonitrile:methanol:water (75:21:14 by vol.) Spm appeared as a shoulder on the solvent front and PC was not retained at all. In acetonitrile:methanol:water (75:15:10 by vol.) PC was not re-

tained but Spm peaked just after the solvent front.

In acetonitrile:methanol:water (75:9:6 by vol.) PC was eluted just after the solvent front and Spm was resolved from the PC peak. LPE in ethanol was also injected and it was resolved from the Spm peak. Figure 5 shows the separation of PC, Spm and LPE.

The acetonitrile was increased to create a ratio of acetonitrile:methanol: water (85:9:6 by vol.) in an attempt to move the PC away from the solvent front. In this system four standards were injected which had the following retention times at flow rate of 1.6 ml/min: PE–1.6 min, Spm–3.4 min, PE–4.2 min and LPE–15.4 min. However when a mixture was injected PE and Spm were unresolved showing only one peak at 3.8 min for both. The solvent system acetonitrile:methanol:water (90:9:6 by vol.) was attempted, but in that case PE, LPC and Spm all eluted together in one unresolved peak. Because PE, LPC and Spm could not be resolved in an acetonitrile:methanol:water solvent system, an alternative system was investigated for the Vydac column.

The Hexane:Isopropanol:Water Solvent System. Geurts Van Kessel *et al.* (40) controlled phospholipid retention times by adjusting the water content in a hexane:isopropanol:water solvent system while maintaining a constant ratio of hexane:isopropanol (6:8 by vol.). In their separation of human erythrocyte membrane lipids they used a gradient of hexane:isopropanol:water (6:8:0.75 to 6:8:1.4 by vol.). From their experience it was felt that a study of the effect of water on PE retention time would help in the design of the system.

The detector was set at 206 nm and the Spectro Physics 3500B two pump system was used to vary the water concentration over a range of 5% up to 16%. Pump A reservoir contained hexane:isopropanol (6:8 by vol.) and pump B reservoir contained water. The column was allowed to equilibrate 15 minutes between each increase in water.

Figure 6 shows the effect of increasing water concentrations on the retention time of PE. As the amount of water increased the retention time decreased and the peak width narrowed. At 11% water the PE appeared as a shoulder on the solvent front.

At 16% water PC, PS, PI, LPE, Spm and LPC all produced very broad peaks

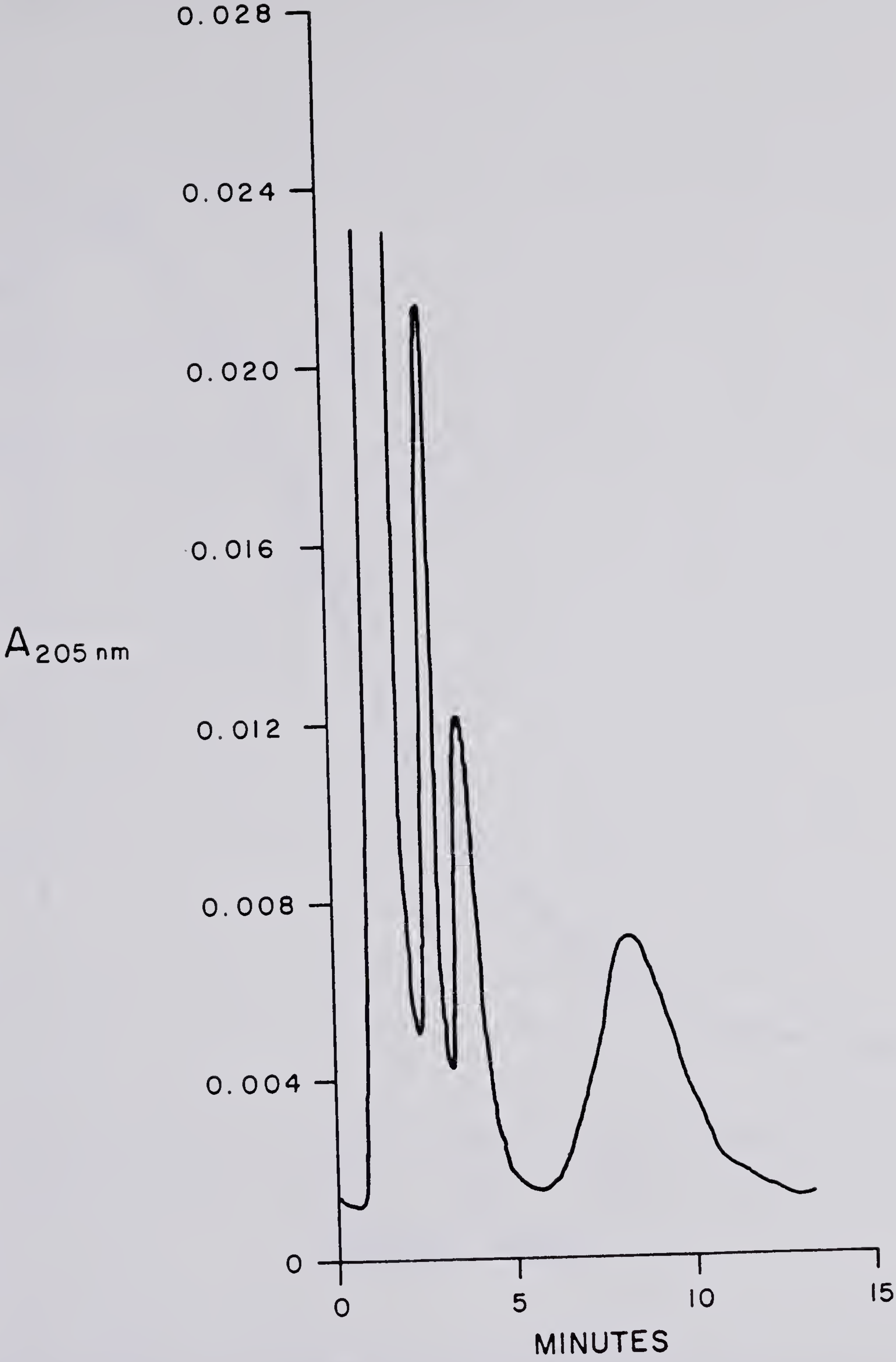


Figure 5. The separation of PC, Spm and LPE. Solvent system, acetonitrile:methanol: water (75:9:6 by vol.); flow rate 0.8 ml/min., room temperature, detection at 205 nm, column, Vydac silica gel 10 μ (500 x 2.0 mm) SF-solvent front, A–PC, B–Spm, and C–LPE.

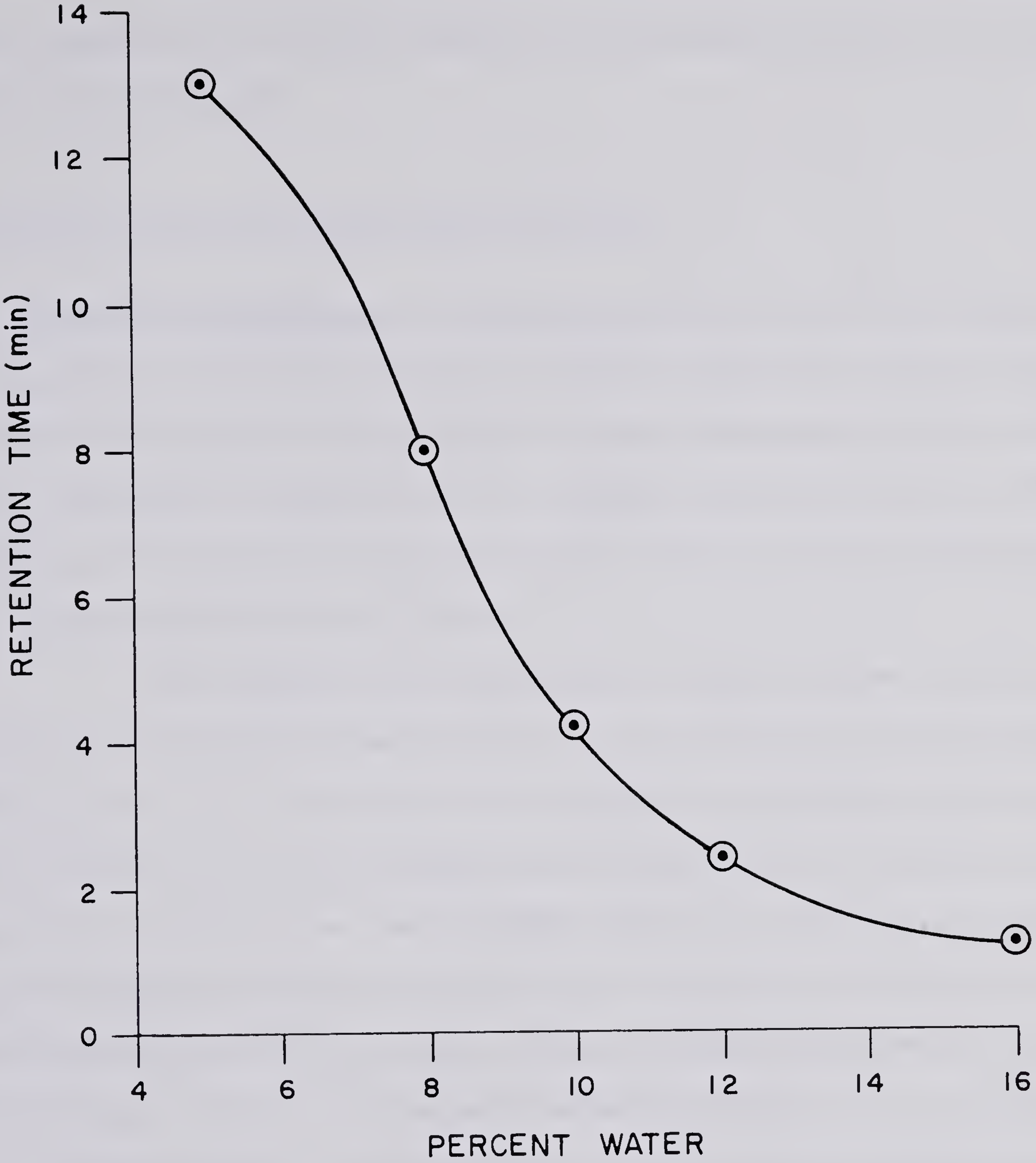


Figure 6. The effect of water concentration on retention time of PE. Solvent system, hexane:isopropanol (6:8 by vol.) and water (5–16%), flow rate 1.0 ml/min., room temperature, detection at 206 nm, column Vydac silica gel 10 μ (500 x 2.0 mm).

with slight variations in retention times. If injected together they would overlap to form one broad peak.

It was concluded that because of the broad nature of the peaks for the standard phospholipids and their poor resolution, the Vydac column was simply not suitable for the study of this system.

Preliminary Studies with the Whatman Silica Gel Column

Evaluation of Solvent Mixtures. A Whatman silica gel 10 μ (500 x 4.6 mm) equipped with a Whatman guard column (2.0 x 70 mm) containing Whatman Pelico silica (30–50 μ) was used in the separation of phospholipids in acetonitrile:methanol:water mixtures. The water concentration was kept at 14% and the acetonitrile:methanol ratio was varied. The flow rate was maintained at 2.0 ml/min during these studies and the column temperature was ambient, the detector was set at 205 nm.

A PE standard was used to determine if PE could be resolved from the solvent front. In acetonitrile:methanol:water (31:55:14 by vol.) PE was not resolved from the solvent front, so no other standards were evaluated. In acetonitrile:methanol:water (45:41:14 by vol.) PE had better resolution from the solvent front but the separations were inadequate. A standard mixture containing DPPE, DPPC, Spm and LPPC was injected, but again PE had poor resolution from the solvent front. The solvent system acetonitrile:methanol:water (60:26:14 by vol.) was found to give peak broadening and long retention times in the mixture separation. In the acetonitrile:methanol:water (55:31:14 by vol.) solvent system adequate resolution was noted but the (51:35:14 by vol.) solvent system gave as good resolution with shorter retention times. The separation of mixture of DPPE, DPPC, Spm and egg-LPC is shown in Figure 7 using acetonitrile:methanol:water (51:35:14 by vol.). Table III shows the retention times of the standard mixture in the four solvent systems. When PS and PI were injected in acetonitrile:methanol:water (51:35:14 by vol.) solvent system and acetonitrile:methanol:water (55:31:14 by vol.) they both eluted in the

Table III

The Effect of Solvent Ratio on Retention Time

Solvent Mixture (%)			Phospholipid Retention Time (min.)			
Acetonitrile	Methanol	Water	DPPE	DPPC	Spm	LPC
45	41	14	4.9	11.8	15.8	18.2
51	35	14	6.0	13.8	17.4	22.6
55	31	14	8.6	17.5	22.4	27.6
60	26	14	16.0	33.0	42.0	59.0

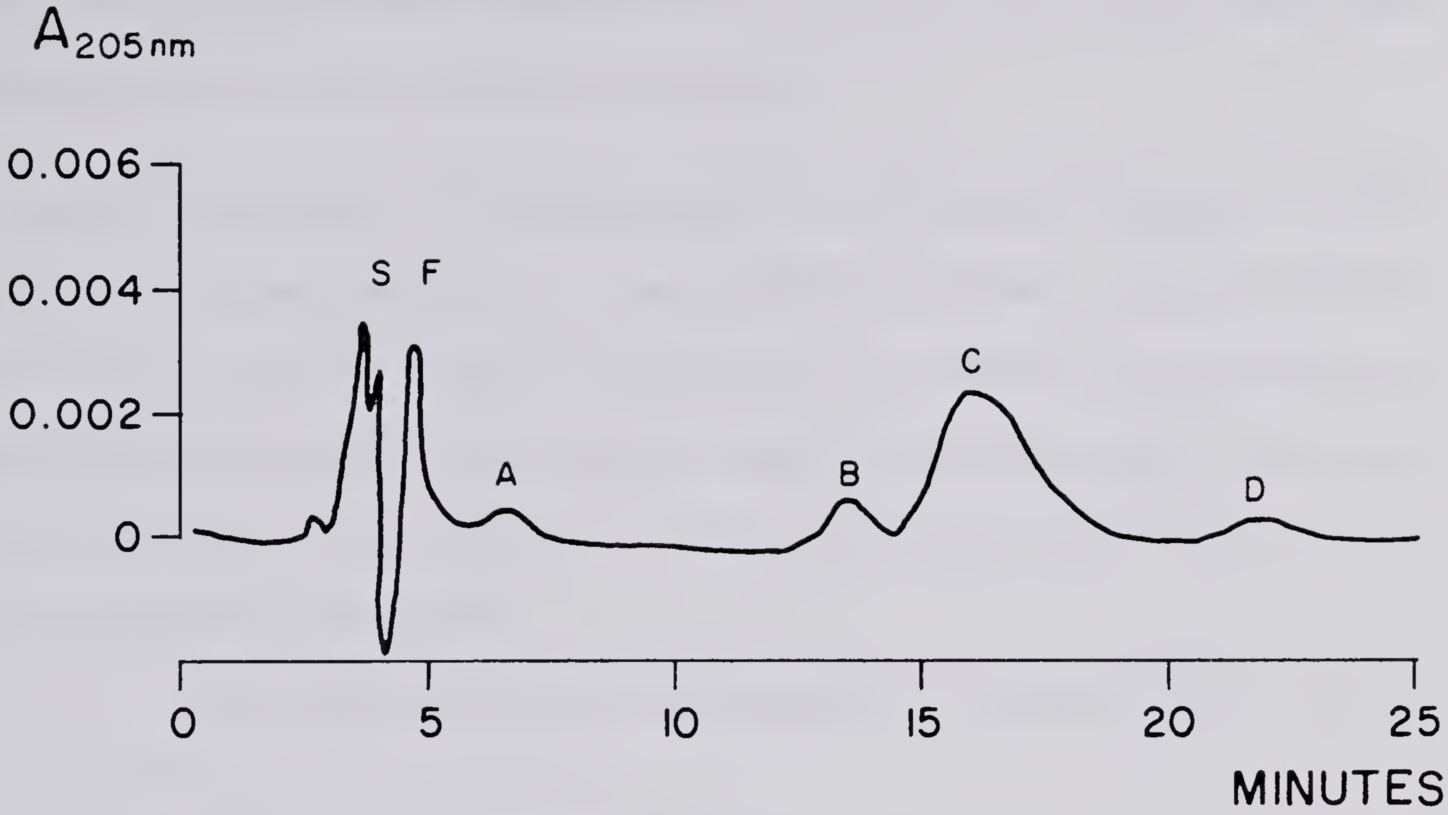


Figure 7. Separation of DPPE, DPPC, Spm and LPC. Solvent system, acetonitrile:methanol:water (51:35:14 by vol.); flow rate 2 ml/min., room temperature, detection at 205 mm, column, Whatman silica gel 10 μ (500 x 4.6 mm), SF-solvent front, A-DPPE, B-DPPC, C-Spm, and D-egg-LPC.

solvent front but PS appeared to partially mask the PE peak just after the solvent front.

Acetonitrile:methanol:water solvents (46:40:14 by vol.), (61:25:14 by vol.) and (28:58:14 by vol.) were all examined using natural phospholipid samples dissolved in ethanol, but they did not give an improved resolution over the (51:35:14 by vol.) solvent system. Hence the acetonitrile:methanol:water (51:35:14 by vol.) was the solvent system chosen to test the separation of natural phospholipid mixtures.

The HPLC Separation of Phospholipids in Natural Phospholipid Extract. The HPLC separation of a phospholipid extract of beef muscle was compared to the separation obtained from natural source standards (PL Biochemical, see Appendix I). Each standard was diluted with HPLC solvent to approximately 1 mg/ml and injected using 10 μl injection loop (approximately 10 μg quantity). This procedure minimized solvent front absorption and ensured solubility of the lipids.

In this study the flow rate was increased to 2.8 ml/min to speed up the analysis. The UV detector was set at 205 nm.

Figure 8 shows UV detector response of the separation of the standards. Figure 9 depicts the separations of extracted beef lipids sample. Table IV shows a comparison of retention times of peaks of the standards and the sample.

There was good retention time agreement between standards and sample peaks except for LPC. This may be the result of different fatty acid chain composition (explained in the next section). The retention times are different from those given in Table III due to the increased flow rate.

Similar elution profiles for both standards and the beef sample were obtained from several other solvent systems. Different flow rates altered retention times proportionally but the order of elution of the peaks remained the same.

Evaluation of standards for possible quantification by UV absorption. Quantification using UV absorption is difficult because of varying extinction coefficients of individual fat-

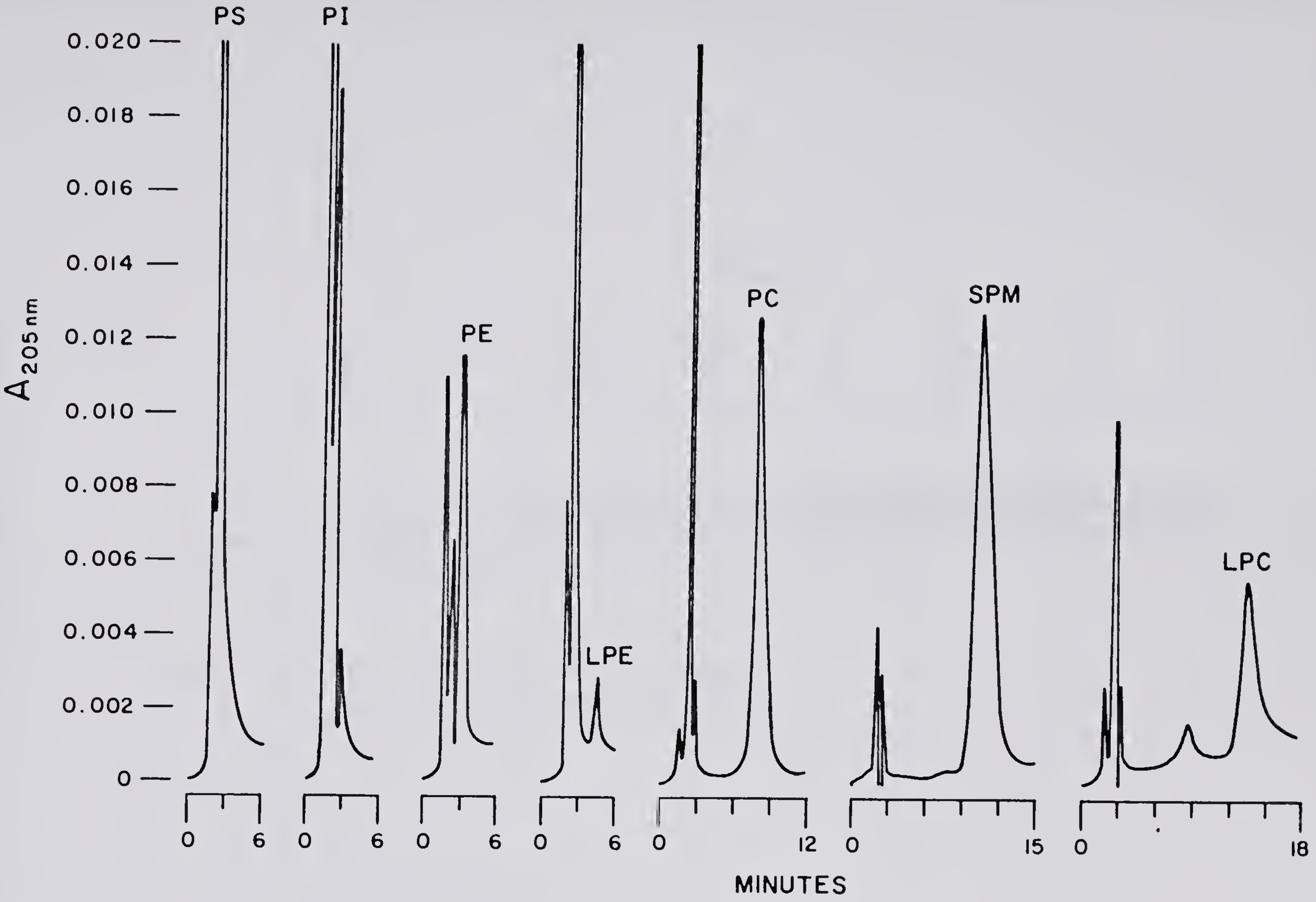


Figure 8. Injection of individual phospholipid standards. Approximately 10 μg injection of PS, PI, PE, LPE, PC, Spm, and LPC, are shown in individual chromatograms. Solvent system; acetonitrile:methanol:water (51:35:14 by vol.) flow rate 2.8 ml/min., room temperature, detection at 205 nm, column, Whatman silica gel 10 μ (500 x 4.6 mm).

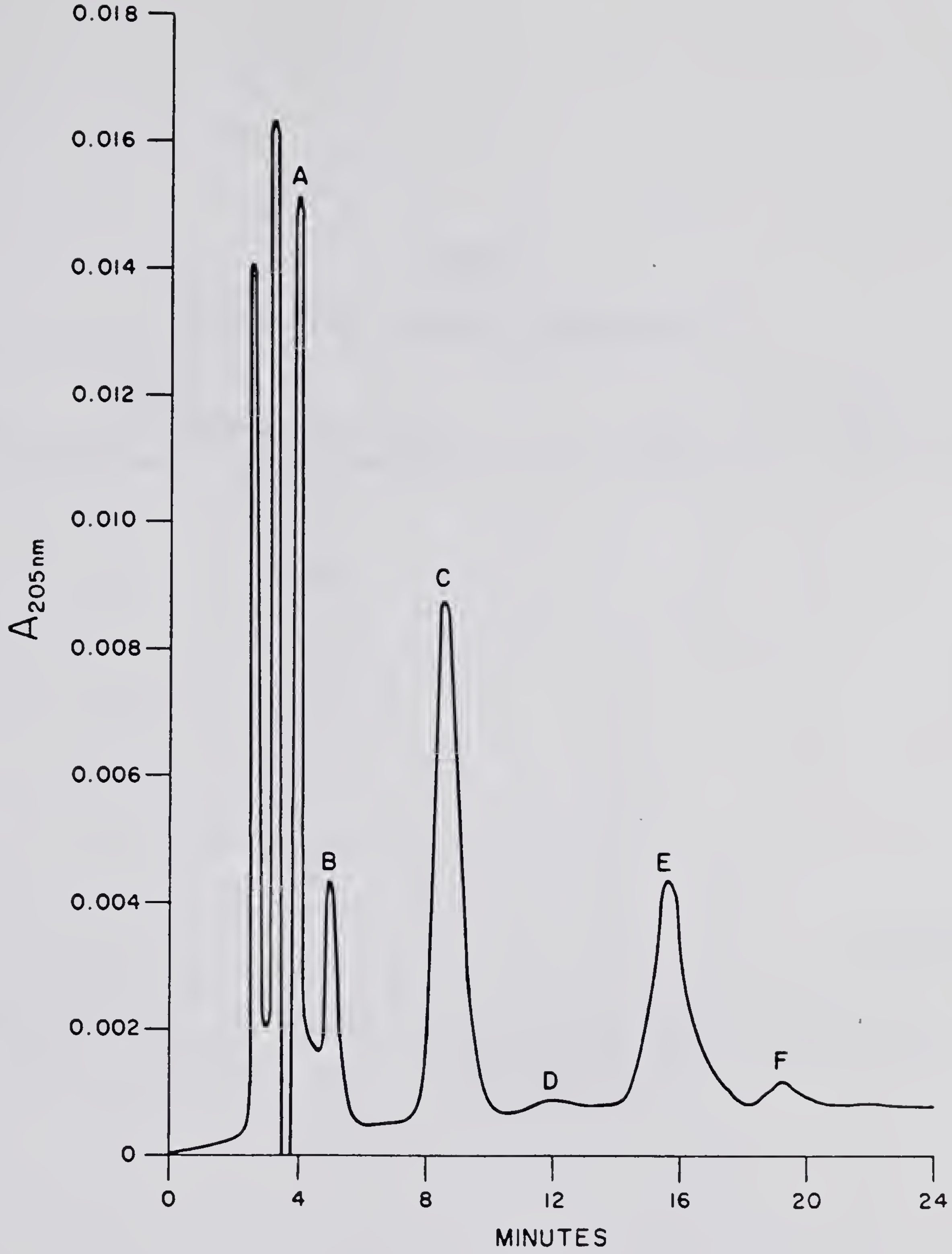


Figure 9. Separation of a beef muscle phospholipid extract. The sample was injected under the same conditions as in Figure 8. Injection (approx. 50 μg). Peaks correspond with standards: A–PE, B–LPE, C–PC, D–Spm, E–LPC, F–unknown.

Table IV

Retention Times of Standards and Sample Separation

Phospholipid	Standards* Retention Time (min.)	Peak	Beef Muscle** Retention Time (min.)
PS	SF		SF
PI	SF		SF
PE	2.9	A	2.9
LPE	4.4	B	4.3
PC	8.3	C	8.3
Spm	11.0	D	11.0
LPC	13.2	E	14.2
		F	unknown 18.5

The standards were injected in duplicate. Conditions outlined Figure 8. SF–Solvent Front.
*From Figure 8.
**From Figure 9.

ty acid chains within a class of phospholipids (38, 40). In order to study this phenomenon four different PCs (DPPC, DSPC, DOPC, DLPC) and three LPCs (1-oleoyl-LPC, egg-LPC, 1-linoleoyl-LPC) were used.

The same chromatographic conditions used for solvent system evaluation were used in this study. The area under the peaks was calculated from the weight of tracings which were carefully cut out of the chart paper. The weight was then divided by 6.15 mg/cm^2 and is presented in cm^2. Table V shows the results of this evaluation. Figure 10 shows the UV response of PCs and Figure 11 shows the UV response of LPCs.

There was significant variation noted in retention times and UV absorption of the three lyso-lecithins and four phosphatidyl cholines evaluated. In order to come to some conclusions about fatty acid chain vs. retention time, a much larger number of lipids would have to be examined, though there appears to be a trend that saturates have longer retention times. The UV response at 205 nm seems to be dependent on the degree of unsaturation. However the relationship is not simple since dilinoleoyl phosphatidyl choline has twelve times the absorption of dioleoyl phosphatidyl choline with only one more unsaturated site. An added complication is the possibility that the more highly unsaturated phospholipid standards may be extensively oxidized. From these results it was concluded that UV absorption is inadequate for the quantification of phospholipids.

DIGESTIVE TECHNIQUES FOR THE DETECTION OF PHOSPHOLIPIDS IN THE HPLC ELUENT

As a result of varying ultraviolet absorption within the phospholipid classes due to differences in fatty acid composition it was necessary to develop an alternative method for the quantification. In TLC separations of phospholipids, quantification is achieved by scraping each spot from the plate, digesting it and subsequently measuring the amount of phosphorus in the digest. Two phosphorus assay methods (a) Morrison (79) and (b) Itaya and Ui (78) as modified by Bowyer and King (71) were examined as possible means of quan-

Table V

Retention Time and UV Response of Phospholipid Types

Phospholipid	Source	Fatty Acid	Retention Time (min.)	Area at 0.08A cm^2 (50 μg)
Dipalmitoyl phosphatidyl Ethanolamine	1	16:0	6.0	1.13
Dipalmitoyl phosphatidyl Choline	1	16:0	14.0	1.15
Distearoyl phosphatidyl Choline	1	18:0	15.2	1.43
Dioleoyl phosphatidyl Choline	2	18:1	12.8	1.53
Dilinoleoyl phosphatidyl	2	18:2	12.2	20.54
Lysolecithin (from egg lecithin phospholipase A)	1	Egg Mixed	23.8	(.99) 1.98
Lysolecithin 1-oleoyl	2	18:1	22.8	(1.02) 2.04
Lysolecithin 1-linoleoyl	2	18:2	24.6	(2.99) 5.98

Phospholipid sources were 1-Sigma, 2-Serdary Research labs. Area under peaks for phospholipids were measured at 0.08A full scale deflection using conditiosn outlined in text. Area was calculated by weighing carefully cut out peaks and dividing by weight of known area (6.15 mg/cm^2). The values in brackets for LPCs are experimental for 25 μg sample. They were doubled for comparison to other values in table.

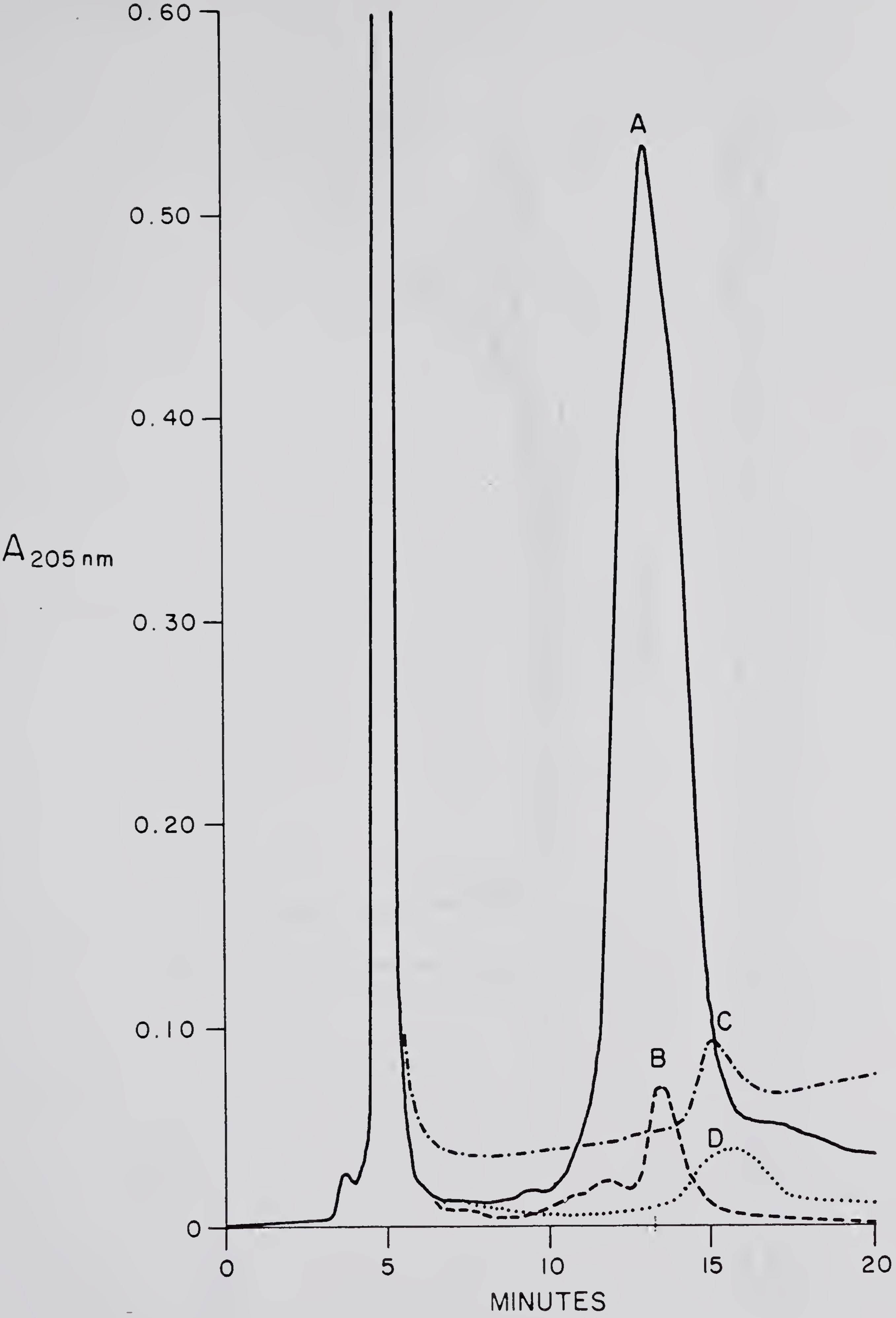


Figure 10. Comparison of peak shape, UV response and retention times of phosphatidyl cholines. Solvent system, acetonitrile:methanol:water (51:35:14 by vol.); flow rate 2 ml/min. room temperature, detection at 205 nm, column, Whatman silica gel 10 μ (500 x 4.6). Injection of 50 μg of A–18:2 PC, B–18:1 PC, C–16:0 PC, and D–18:0 PC.

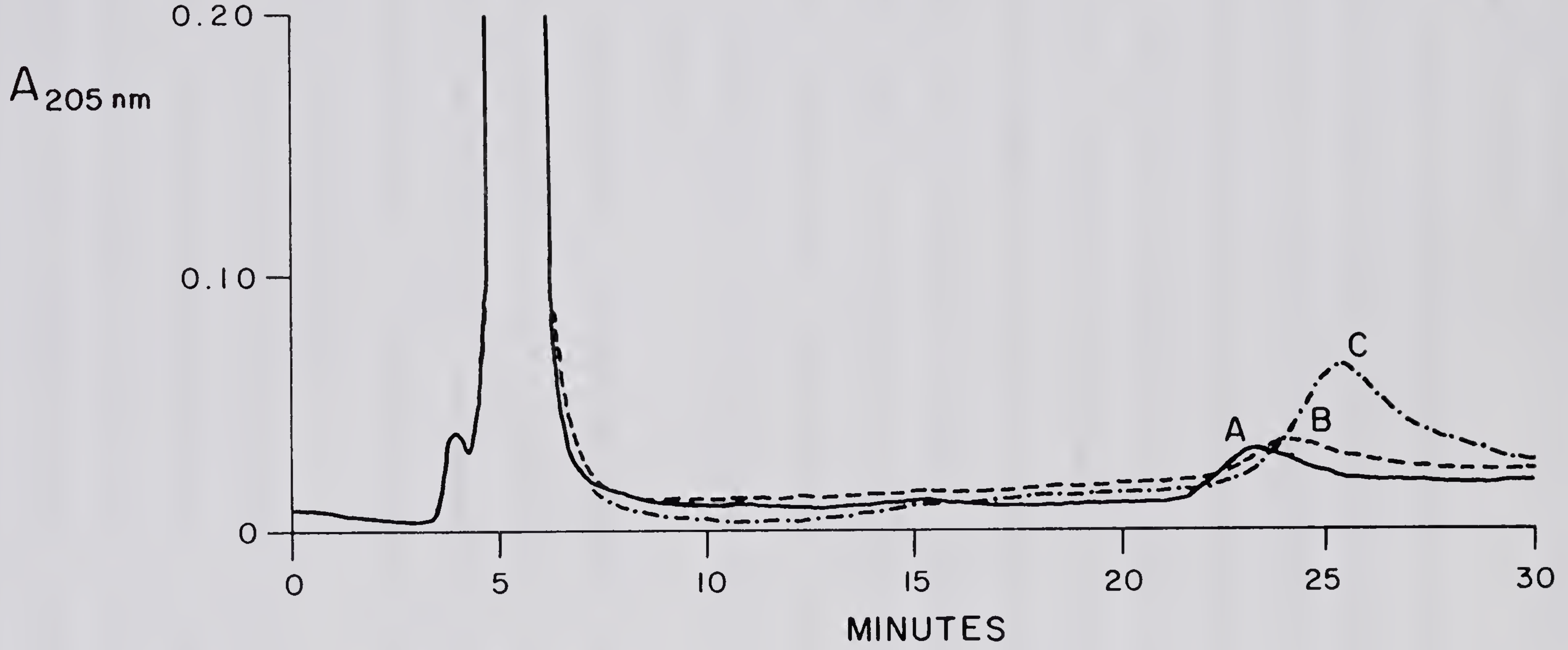


Figure 11. Comparison of peak shape, UV response and retention times of lysophosphatidyl choline. Solvent system, acetonitrile: methanol:water (51:35:14 by vol.); flow rate 2 ml/min. room temperature, detection at 205 nm, column, Whatman silica gel 10 μ (500 x 4.6 mm). Injection of 25 μg of A–1-oleoyl-LPC, B–egg-LPC, C–1-linoleoyl-LPC.

tifying the phosphorus in the eluent fractions.

The Morrison technique (79) was found to be inadequate as it was not sensitive enough for the quantification of the minor constituents of phospholipids in the eluent of a separation of HPLC.

The Itaya and Ui (78) phosphorus assay, based on the principle that malachite green at low pH forms a complex with phosphomolybdate, is sensitive in the range of 0.05 to 1 μg phosphorus or 1 μg to 25 μg phospholipid. Bowyer and King (71) modified the technique by decreasing the volume and thereby increased the sensitivity.

The procedure was carried out as outlined by Bowyer and King (71). Unwashed test tubes 12 x 75 mm were used in the analysis. All reagents were made up in doubly distilled water and glassware was cleaned in chromate before use, no detergents were used. A standard curve for phosphorus using Baker Analytical grade KH_2PO_4 (which was dried overnight at 110° C) was measured over a range of 0.05 to 1.00 μg phosphorus (as shown in Figure 12) using the small volume technique. Also a phospholipid extract (beef) was analyzed to check digestion efficiency and linearity over a range 2 to 20 μg phospholipid as shown in Figure 13.

A total lipid extract of beef was analyzed by HPLC within twelve hours of the extraction. A 1 ml sample in chloroform:methanol (2:1 by vol.) containing 22.4 mg/ml (weighed by Cahn Gram Electrobalance) was evaporated to dryness with N_2 and redissolved in 0.5 ml of ethanol for injection. The injection volume was 10 μl which contained approximately 450 μg of total phospholipid extract.

The solvent system used was acetonitrile:methanol:water (51:35:14 by vol.) at a flow rate of 3 ml/min. Fractions were collected at 12 second intervals and evaporated to dryness with N_2 prior to analysis. Fraction numbers 17 and 18 and 38 through 42 were analyzed using twice the volume of reagents because the phospholipid concentrations were too high to measure using the small volume technique. From the two analyses undertaken it was found that the controls were very high (0.151A at 660 nm) and it is therefore recom-

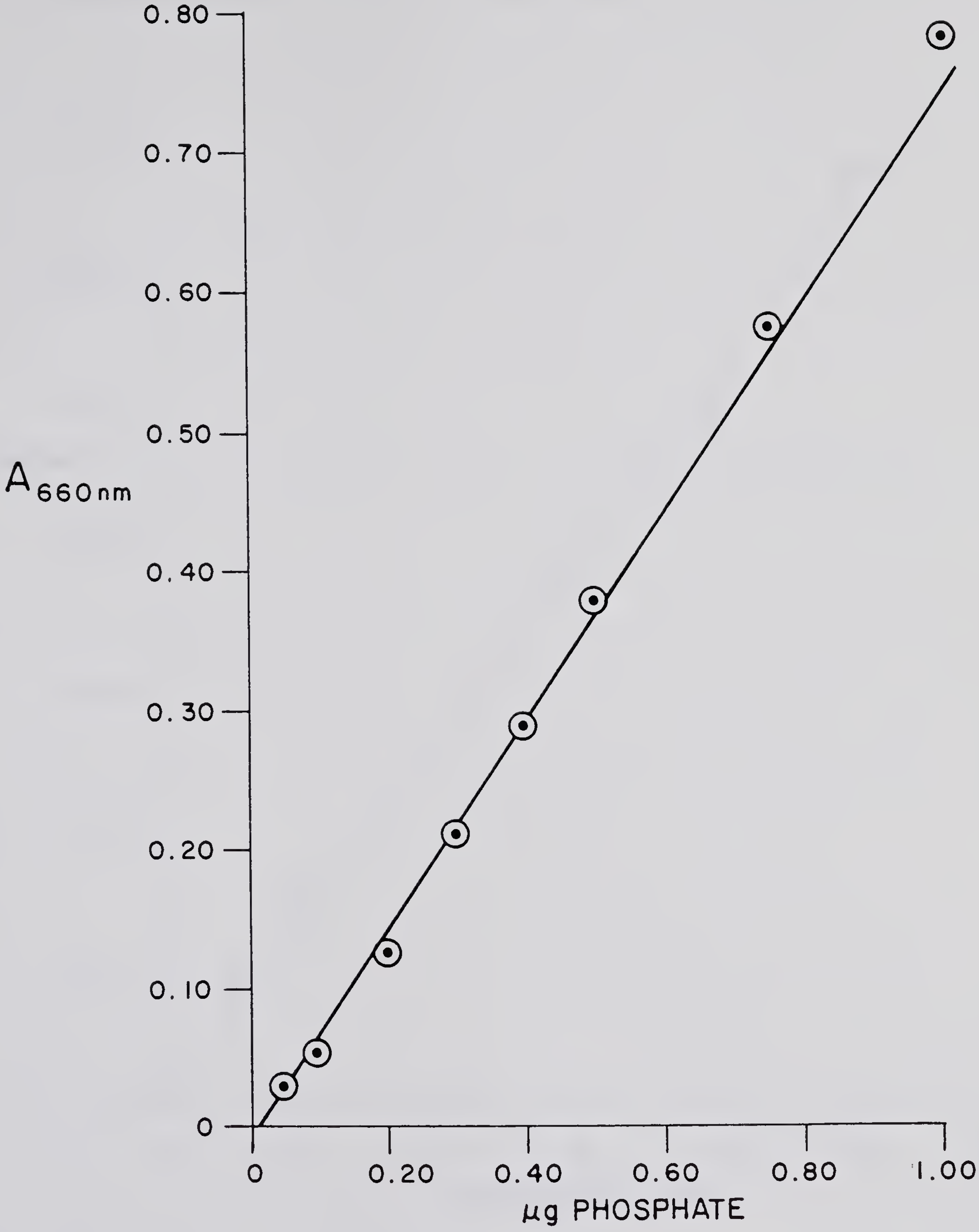


Figure 12. The analysis of inorganic phosphate by the Bowyer and King (71) method.

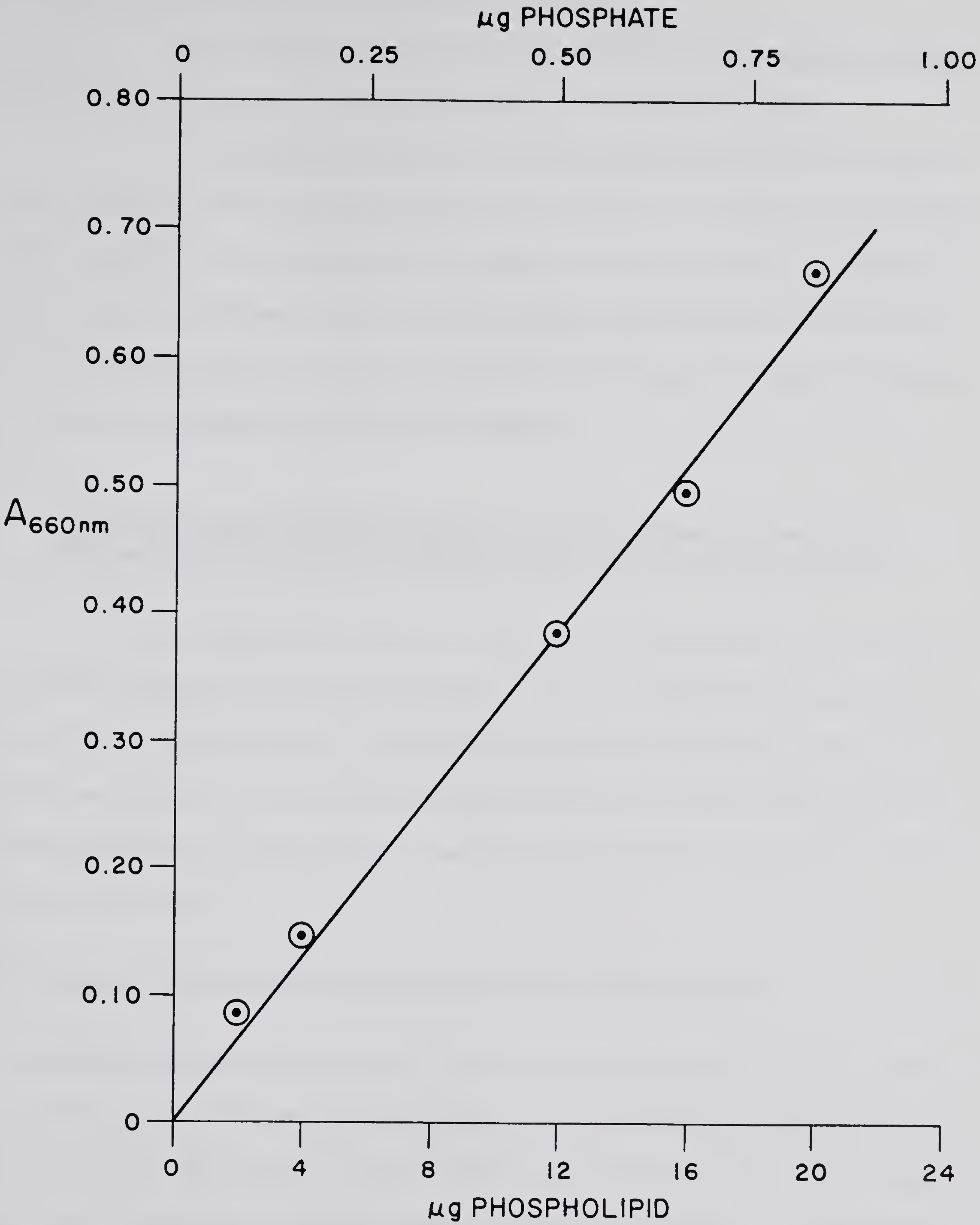
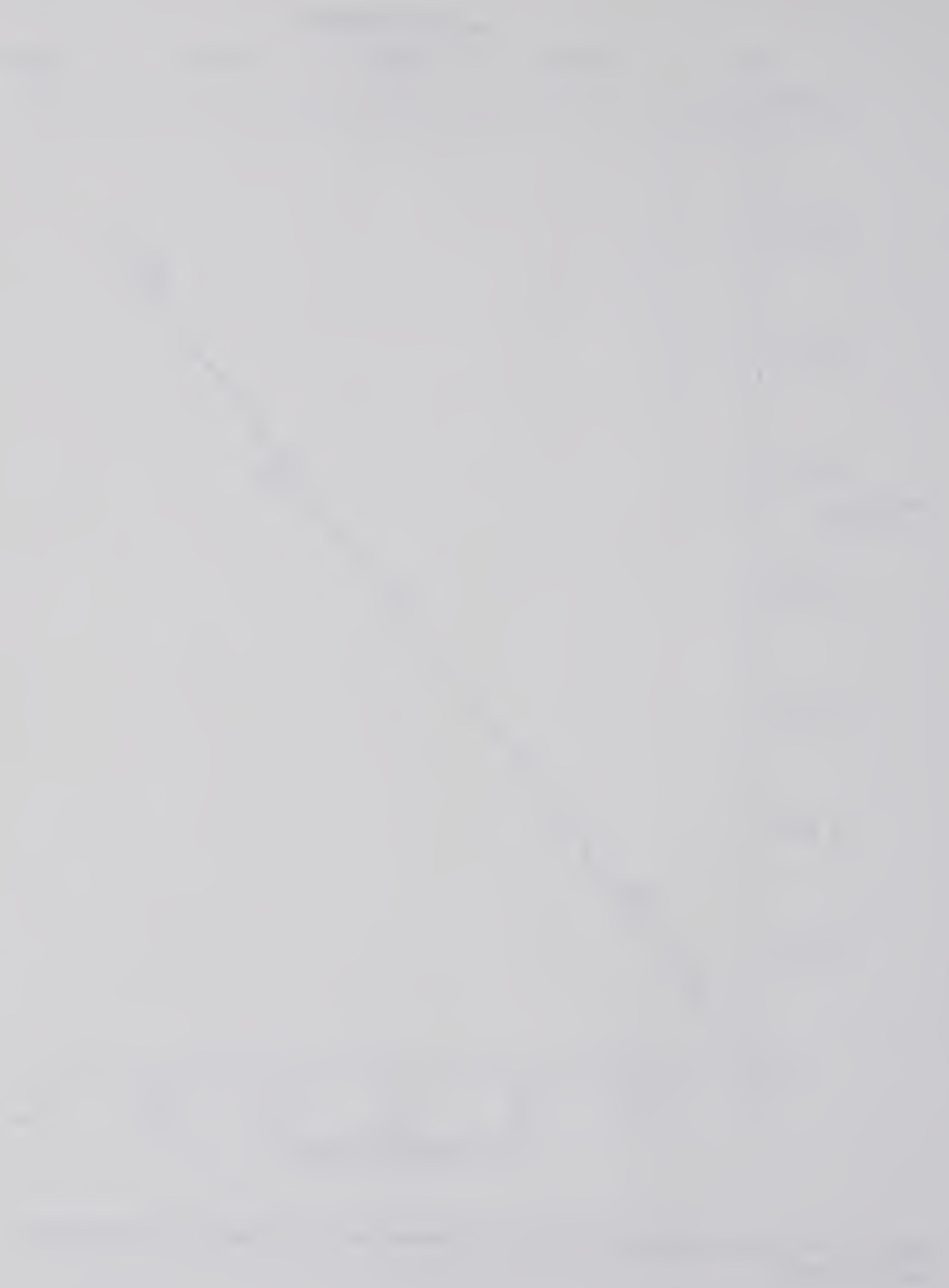


Figure 13. The analysis of inorganic phosphate from phospholipids after acid digestion by the Bowyer and King (71) method.



mended that the test tubes be acid washed before analysis.

Figure 14 shows the simultaneous UV monitoring and phosphorus analysis. There is good peak agreement between UV detector and the phosphorus analysis.

For this preliminary study a perchloric acid fumehood was used for the digestion because of the risk of explosion from peroxide formation. The Spectro Physics 3500B HPLC was used for the separation and the phosphorus analysis was carried out in the Food Science Department. It was concluded that for routine analysis this assay was far too tedious. The digestions had to be done in a perchloric acid fumehood as outlined by McClare (72) which is not present in the Food Science Building.

THE DEVELOPMENT OF NON-DIGESTIVE COLORIMETRIC TECHNIQUES FOR THE MEASUREMENT OF PHOSPHOLIPIDS AFTER HPLC SEPARATION

Since ultraviolet detection as a method of quantification of phospholipids after HPLC separation was inadequate because it cannot be calibrated and digestive techniques are very tedious and not very amenable to automation, the research reported in this section was undertaken to develop a non-digestive colorimetric technique based on the techniques presently in use. Experimentation was done with the direct adaptation of a method for automation in mind.

The Transfer of Vaskovsky and Kostetsky Spray Reagent by Phospholipids

Immiscibility test with HPLC solvent. Equal volumes of the HPLC solvent acetonitrile:methanol:water (51:35:14 by vol.) and selected extractants were thoroughly mixed on a vortex mixer and then allowed to stand to check phase separations. Hexane and heptane were found to have excellent phase separation qualities at a ratio (1:1 by vol.) with the HPLC solvent. Pentane and isopentane also exhibited phase separation; but not as rapidly as hexane and heptane. Chloroform, dioxane, isobutanol, 2-butanol, 3-methyl-1-butanol, isopropanol, octanol, dimethyl sulfoxide were miscible with the HPLC solvent. Benzene and

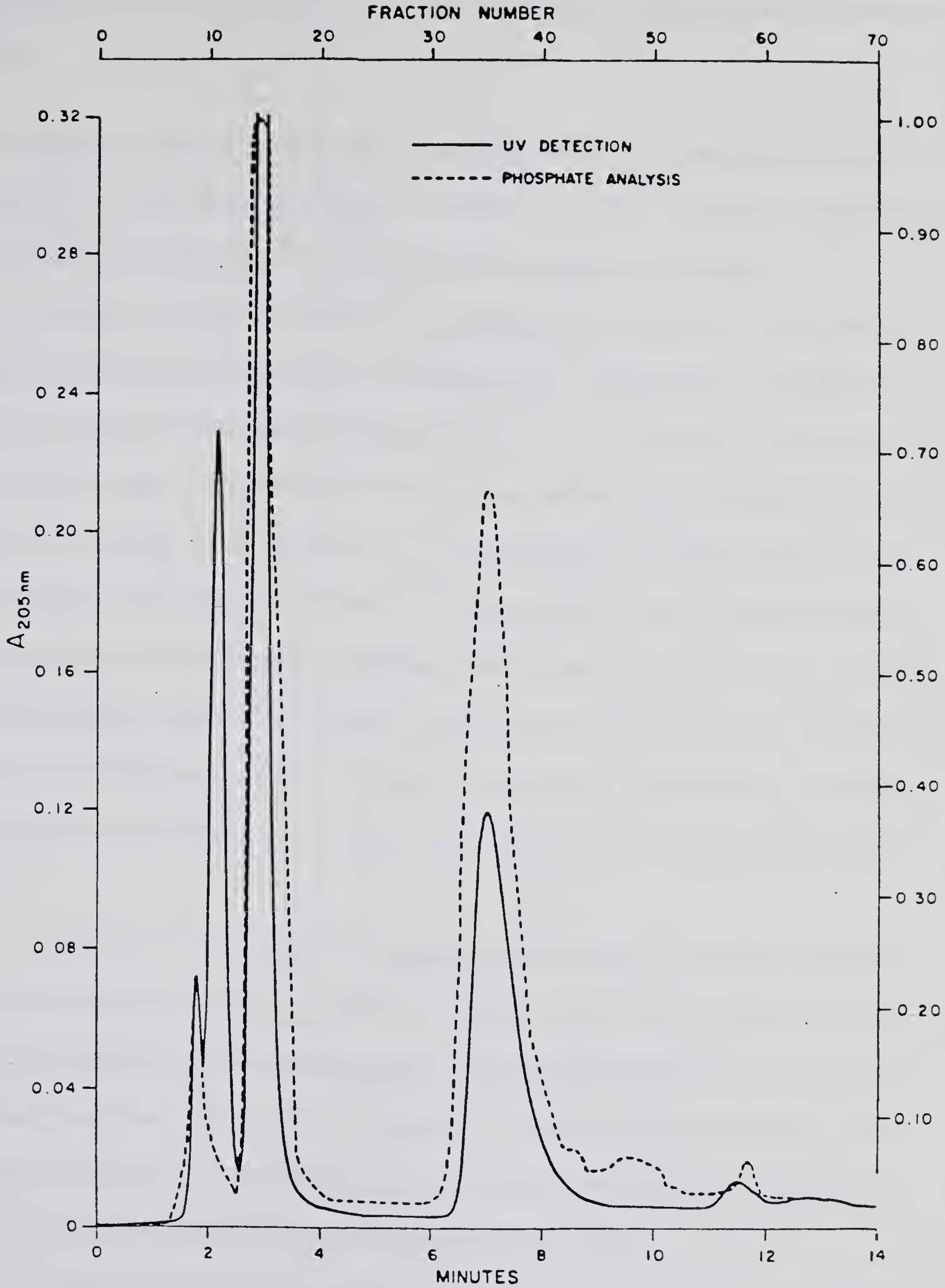


Figure 14. A comparison of UV detection and inorganic phosphate analysis of a beef muscle phospholipid extract after HPLC separation. Chromatographic conditions outlined in text. Inorganic phosphate analysis by Bowyer and King (71) method.

toluene did not mix but the top layer was turbid, so they were initially rejected for use as extractants.

Color extraction experiments using hexane and heptane. The Vaskovsky and Kostetsky spray reagent (95) was produced as outlined by Raheja *et al.* (89). From the immiscibility studies n-heptane was the first solvent used for extraction of the color complex.

Aliquots (0.050 to 0.400 ml) of a phospholipid extract of beef (7.5 mg/ml) were placed in test tubes and evaporated to dryness with N_2. Subsequently the residue was resuspended in 1.5 ml of acetonitrile:methanol:water (51:35:14 by vol.). To each sample 0.3 ml of the spray reagent was added and the mixture was heated in a boiling water bath for 1 minute. The samples were cooled and 1.5 ml of n-heptane (redistilled reagent grade) was added to each. The mixture was mixed on the vortex mixer for 10 seconds. After phase separation took place, 1.3 ml of the upper phase was removed and diluted with 1.0 ml of n-heptane. The optical density of the diluted upper phase was then measured at 710 nm in the Gilford Spectrophotometer. Figure 15 shows the response of phospholipid concentration vs. absorbance of the extracted complex. The deviation from linearity is due to turbidity.

Hexane was also examined as an extracting solvent for the color complex because it had the same phase separation qualities as heptane and is more commonly used in lipid chemistry (110). HPLC grade hexane was tested for the extraction of phospholipid-color complexes with and without heat. It was found that the transfer of the colored complex took place without heating. Therefore, the heating step was eliminated during subsequent testing of the DPPC (Sigma) standard for quantitative transfer.

Aliquots of DPPC (5 mg/ml) in chloroform:methanol (2:1 by vol.) were placed in test tubes in aliquots representing 50, 100, 150, 200 and 250 μg. They were evaporated to dryness with N_2. One milliliter of acetonitrile:methanol:water (51:35:14 by vol.) was added to redissolve the lipid. Then 0.15 ml of color reagent was added, and the sample was mixed on the vortex mixer. 3 ml of hexane was added, the sample was mixed

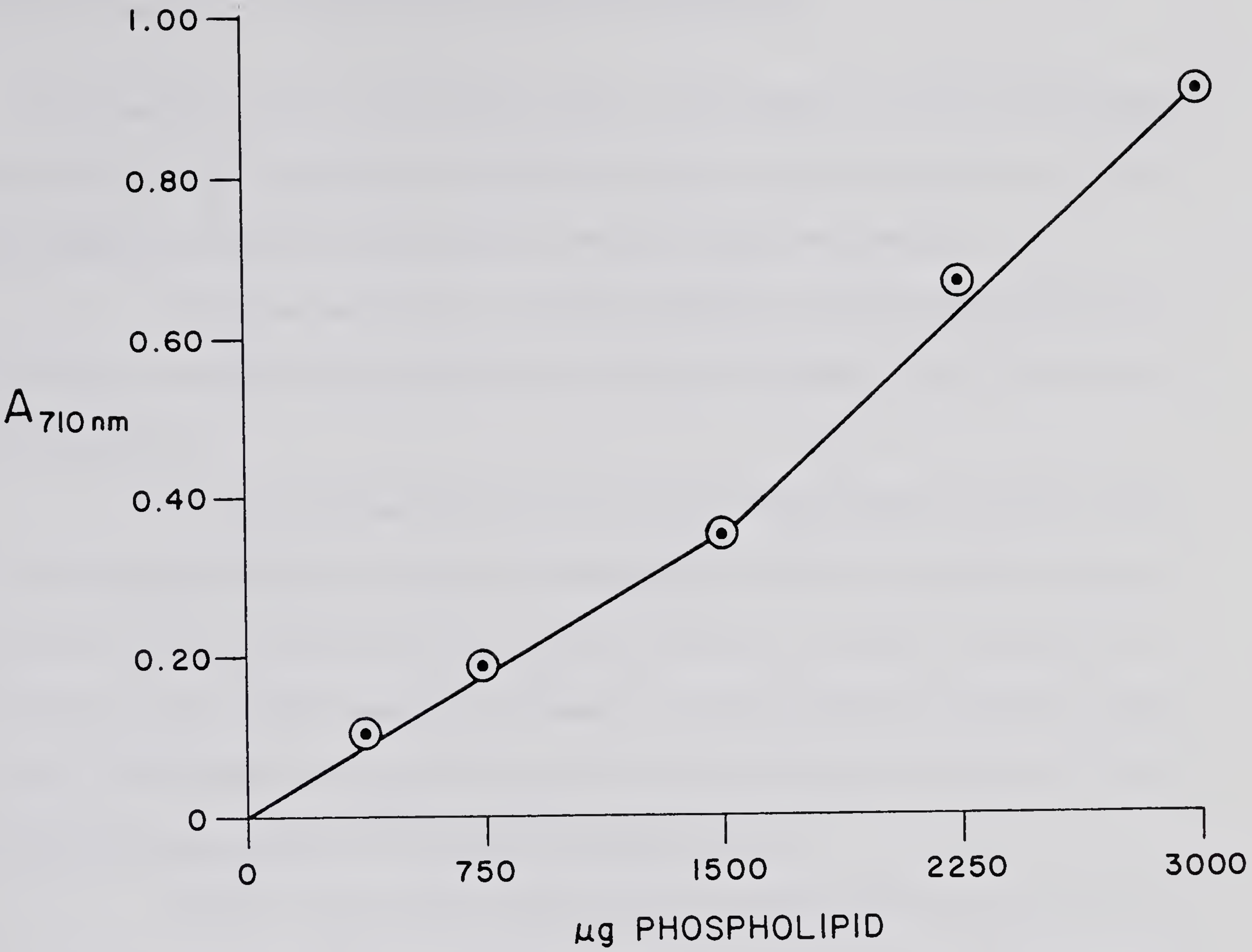


Figure 15. The extraction of phospholipid-molybdate color complex from HPLC eluent by heptane.

again for 10 seconds and allowed to phase separate (almost instantaneous). The top layer was removed and the absorbance was read at 710 nm in a 1 cm microcuvette in the Gilford Spectrophotometer. The standard curve is shown in Figure 16, and is linear from 50–250 μg so hexane was used in subsequent color extraction experiments.

The effect of mercury concentration on color transfer. Mercury acts as a reducing agent in the reduction of ammonium molybdate for the Mo^V state (99). Because of the cost of this reagent the effect of its concentration in the color reagent was evaluated.

Solutions were made as outlined by Raheja *et al.* (89), but the mercury concentration was varied in three solutions [(a) 10 grams Hg (original), (b) 5 grams Hg, and (c) 2 grams Hg)].

Three concentrations 50 μg, 100 μg, and 250 μg of DSPC (5 mg/ml in ethanol) were used to evaluate each reagent. To each sample of 1.00 ml of acetonitrile:methanol: water (51:35:14 by vol.) and 0.15 ml of (a), (b) and (c) were added. The samples were mixed and 2 ml of hexane was added. The samples were then remixed and allowed to phase separate. The absorbance was then measured in the Gilford Spectrophotometer at 740 nm which is the peak maximum for the colored complex in hexane.

Figure 17 shows the results of this experiment. Solution B was chosen as the standard composition to be used because it gave the highest response. The formula is outlined below for a double batch. 32 g of ammonium molybdate is dissolved in 240 ml of water to give solution I. 80 ml of concentrated HCl and 10 ml mercury are shaken with 160 ml of solution I for 30 minutes to give, after filtration, solution II. 400 ml of concentrated H_2SO_4 is then added carefully to the remainder of solution I to prepare the resultant solution III. Solution III was used as the color reagent.

In subsequent experiments it was found that hexane did not adequately transfer DPPE, PI, PS, LPC, LPE and DPG. Hence it was again necessary to try to find a better extraction solvent.

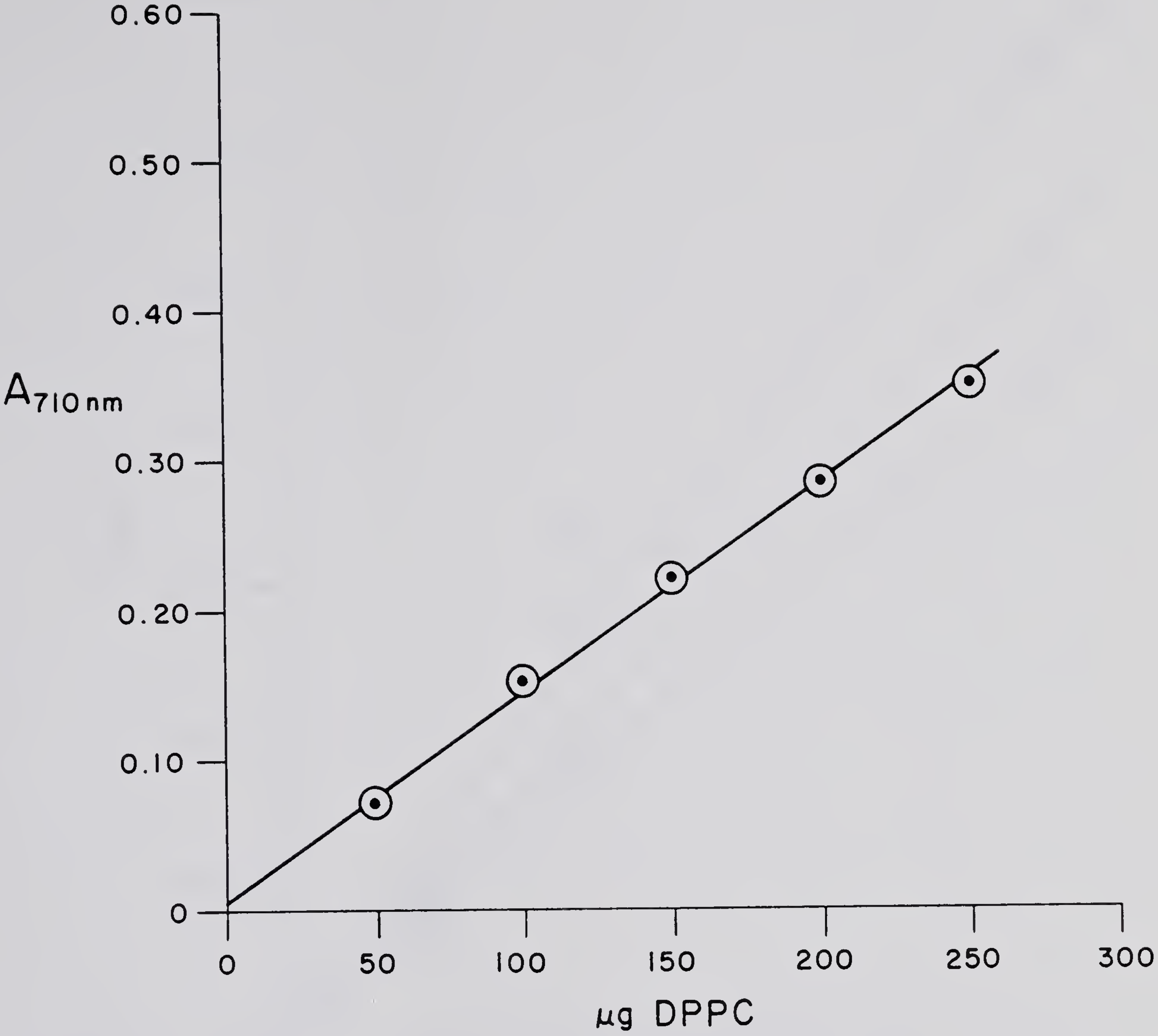


Figure 16. The extraction of DPPC-molybdate color complex from HPLC eluent by hexane.

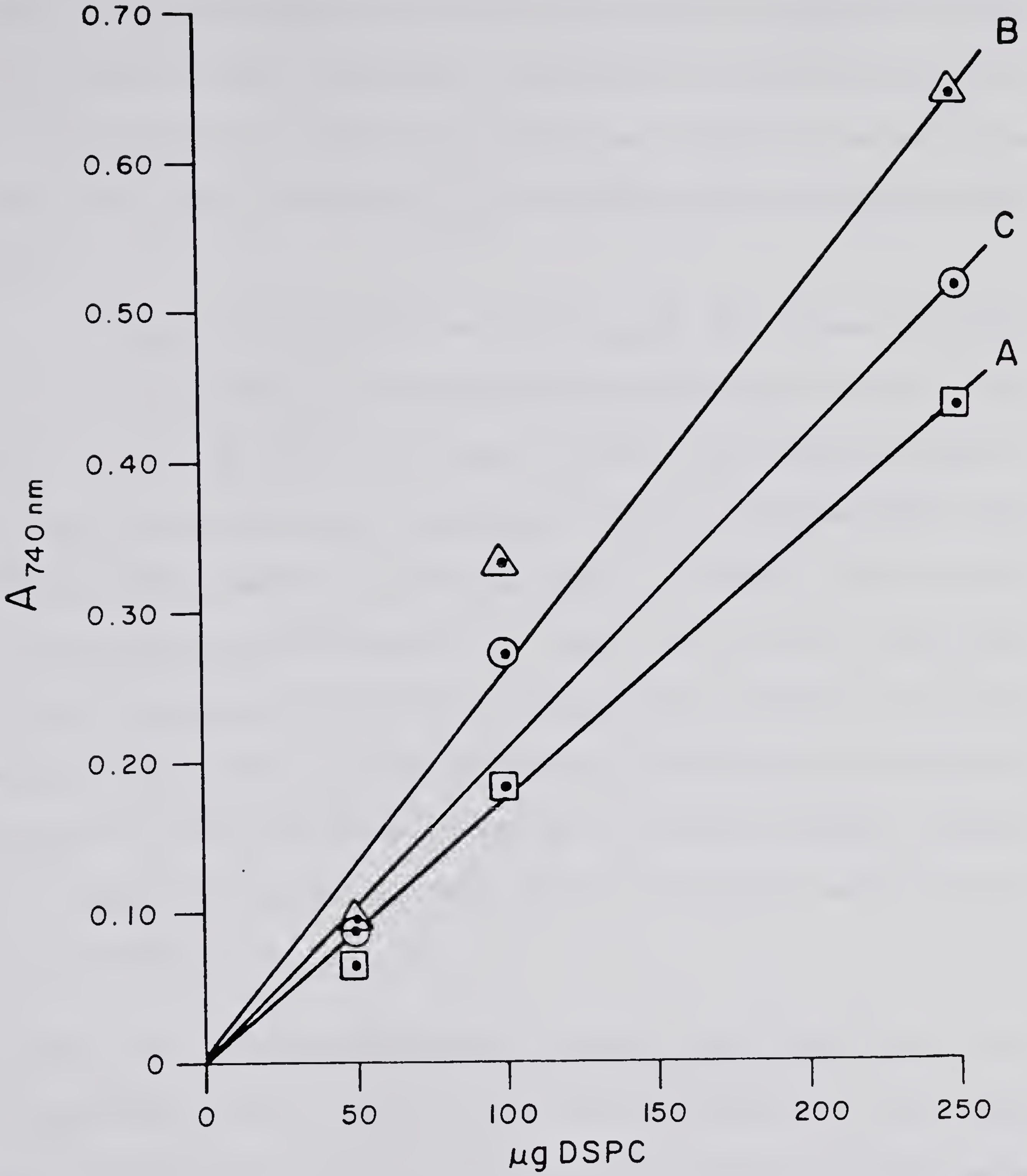


Figure 17. The effect of mercury on color transfer. Transfer was tested using DSPC for a) 10 grams Hg, b) 5 grams Hg and c) 2.5 grams Hg in the formulation of the Vaskovsky and Kostetsky spray reagent (95).

Choosing a solvent for extraction of the colored complex. Chloroform, n-butanol, benzene, toluene and mixtures of hexane:toluene were evaluated as solvents for extraction of phospholipid molybdate complexes. Chloroform and n-butanol were immiscible with the HPLC eluent unless the water concentration exceeded 60% in the HPLC solvent. Even if phase separation took place the blank was very high unless centrifugation was used to clear the turbidity of the solution. Benzene also had the turbidity problem but transfer of DPPE was possible.

Hexane transferred DPPE poorly but egg yolk PE was shown to be transferred. Hexane has the advantage of excellent phase separation. Toluene was tried; it caused turbidity in the upper layer but it did transfer PE adequately. Therefore, mixtures of hexane:toluene were studied using a series of tubes (100% hexane through 100% toluene in increments of 10%) examined by eye for color transfer and turbidity. From this experiment it was concluded that 60% hexane and 40% toluene was the mixture in which maximum toluene was present and transfer still took place without turbidity in the top layer. The maximum transfer point was not the most important criteria because the system was being designed for automation. It was thought that the maximum sensitivity would be achieved by measuring the top layer, therefore in an automated system it would be more important to avoid turbidity.

The effect of color reagent concentration on extraction of the colored complex. Soya lecithin phospholipid extract was used to evaluate the concentration of the color reagent on transfer of phospholipid-molybdate complexes. A series of samples containing varying amounts of color reagent (0.050–0.300 ml) in 1 ml of acetonitrile:methanol:water (51:35:14 by vol.) with 450 μg phospholipid were tested. The color complex was extracted into 2 ml of hexane:toluene (60:40 by vol.) and the absorbance was measured at 740 nm in the Gilford Spectrophotometer.

Figure 18 shows the results of these trials. Optimum concentration of the color reagent (0.150 ml) was used for subsequent experiments. In early experiments, when

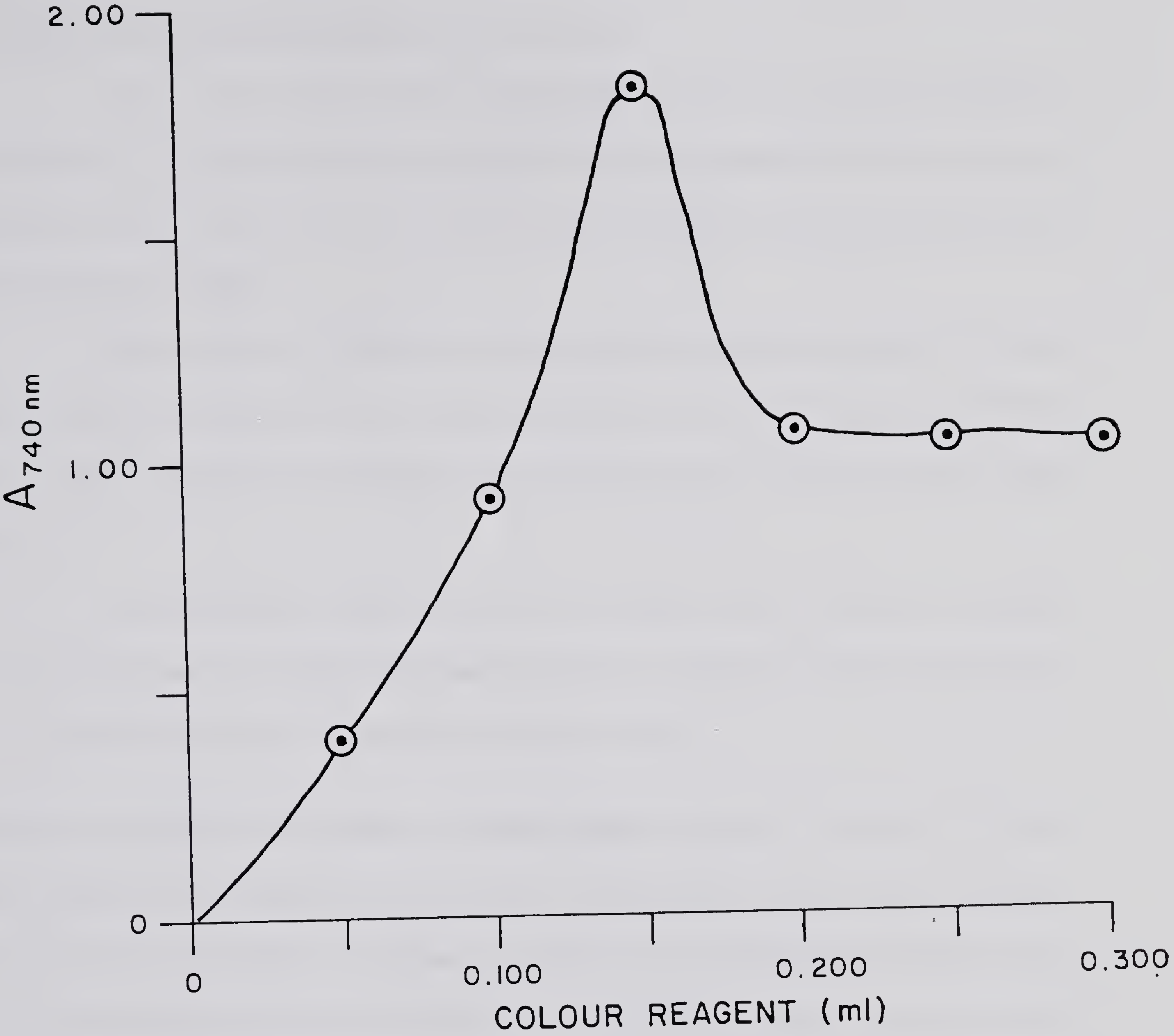


Figure 18. The effect of color reagent concentration on extraction of the colored complex. 450 μg PL from soya lecithin phospholipid extract was added to 1 ml of HPLC solvent. Varying amounts of color reagent were added to the solution and the colored complex formed was extracted with 2 ml hexane:toluene (60:40 by vol.), and its absorbance read at 740 nm.

evaluating toluene, 0.150 ml was also found to be the optimum.

The effect of acid and NaCl on the transfer of color reagent by phospholipids. The effect of H_2SO_4 and NaCl were evaluated. From earlier work in the automated benzene system, H_2SO_4 was known to be necessary for PE transfer.

To 45 μg of soya lecithin phospholipid extract in 1 ml of acetonitrile:methanol:water (51:35:14 by vol.) varying amounts of acid were added (0.020–0.100 ml of concentrated H_2SO_4). Only 0.100 ml of color reagent was added to determine if it was the acid that helped in transfer.

From Figure 19, 0.040 ml was found to be the optimum quantity of H_2SO_4 which would give a cumulative acid concentration close to that of the addition of 0.150 ml of color reagent. Therefore no additional acid was added when 0.150 ml of color reagent was used.

Using the same conditions as above (constant color 0.150 ml, no acid addition) varying amounts of 1 M NaCl were added. Between 0.01 and 0.1 M in the final mixture, NaCl had little effect on the transfer of the color reagent.

Standard conditions of evaluation and phospholipid response. In order to determine reproducibility, soya lecithin phospholipid extract was evaluated under varying conditions. For best results the following conditions were used; 1 ml of acetonitrile:methanol:water (51:35:14 by vol.) with addition of 0.150 ml color reagent; extracted with 3 ml of (60:40 by vol.) hexane:toluene (2 ml if a microcuvette is used) and vortexed 10 seconds. The sample was then heated in a water bath (at 50° C) for 5 minutes, cooled and centrifuged at high speed in the bench top centrifuge. The absorbance of the top layer was measured in 1 cm cuvette at 740 nm in the Pye-Unican SP-8-100 UV Spectrophotometer.

Under these conditions PE, PC, LPC, DPC, PI, PS were evaluated for transfer of reagent complex. The standard curves are shown in Figure 20.

PC and Spm gave the maximum transfer of the phospholipid-molybdate

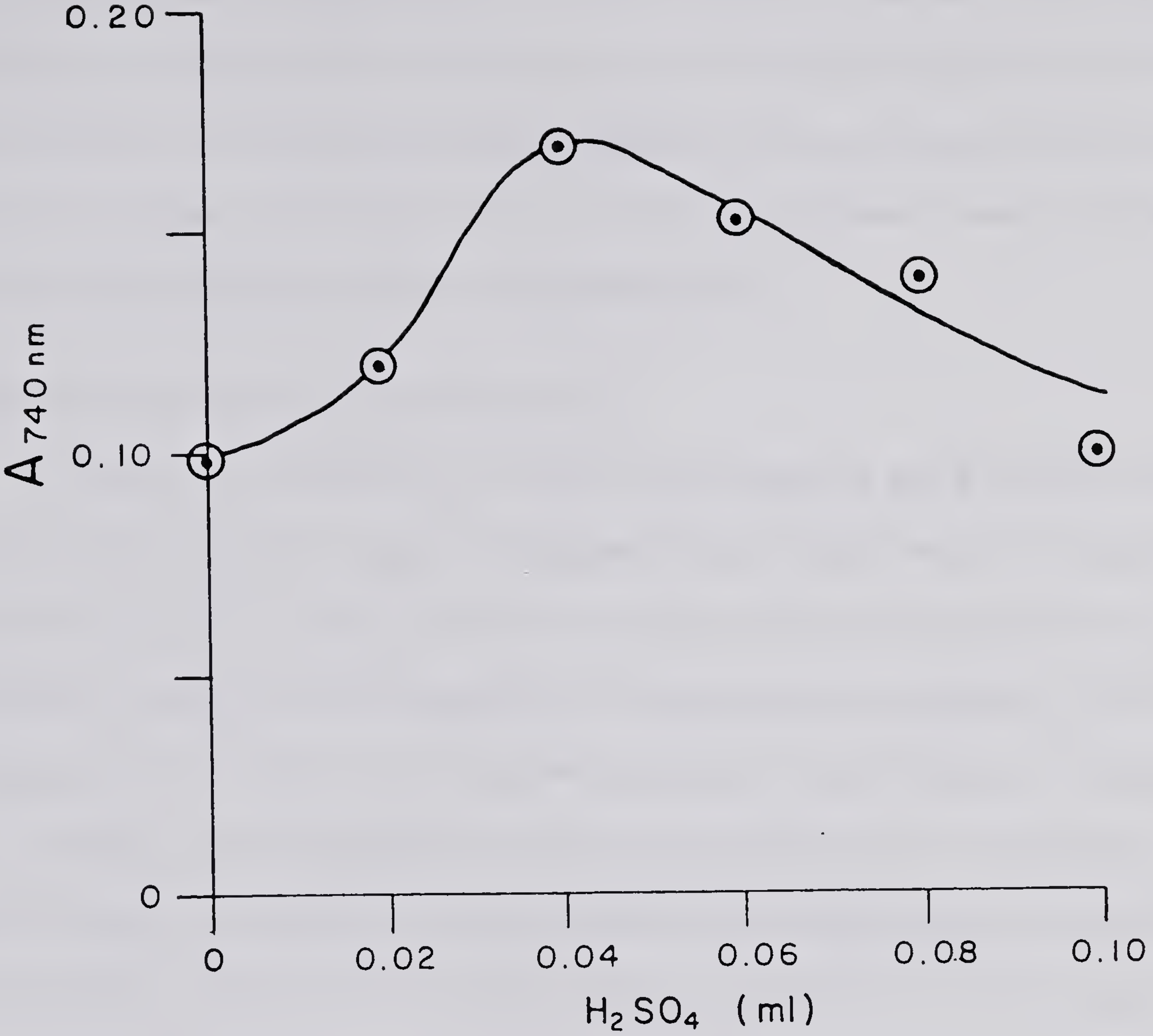


Figure 19. The effect of acid on the transfer of color reagent by phospholipids. 45 μg PL from soya lecithin phospholipid extract was added to 1 ml of HPLC solvent plus 0.10 ml of color reagent. Varying amounts of acid was added to the solution and the colored complex formed was extracted with 2 ml hexane:toluene (60:40 by vol.).

complex while PE, PI and PS show much lower transfer. Under these conditions LPE and LPC transfer was not detected.

From Figure 20 one can see that the Vaskovsky and Kostetsky spray reagent (95) can be used to measure phospholipids in the HPLC eluent by a manual analysis. This reagent would be especially useful in the measurement of PC and Spm which have the highest response. However, from attempts at automating this system it was found that the color reagent was too corrosive on the instrumentation. At this point experiments were undertaken to develop a more satisfactory reagent for automated analysis.

The Transfer of Reduced Molybdate by Phospholipids

Because of the expense and acidity of the Vaskovsky and Kostetsky spray reagent (95), an alternative color reagent was desired. It was thought that the transfer of reduced molybdate should be studied. Galanos (88) observed that both reduced and unreduced molybdate complexed with phospholipids. He first reacted the molybdate with the phospholipid and then reduced it. In preliminary experiments, I have found that reduced molybdate complexes with phospholipids, so the reduction does not have to take place in the presence of lipid. This suggested that the mechanism was possibly based on the formation of an association complex. Based on these criteria the production of a stable color reagent for the transfer of organic solvent by phospholipids was attempted.

Preliminary experiment. To test the system initially 1.0 ml of aminonaphthol sulphonic acid (ANSA) solution and 1.0 ml ammonium molybdate (AM) (4 grams in 30 ml distilled water and 10 ml HCl) were mixed and heated to a boil. The final color reagent consisted of the above mixture diluted with distilled water 25 times. 0.050 ml of this diluted color reagent was mixed with 1.0 ml acetonitrile:methanol:water (51:35:14 by vol.) containing 125 μg DSPC (0.025 ml of 5 mg/ml in ethanol). The sample was then extracted with 2 ml hexane:toluene (60:40 by vol.) and the absorbance was read at 740 nm in the Gilford Spectrophotometer in a 1 cm microcuvette. The results were 0.338A for sample and 0.035A for control.

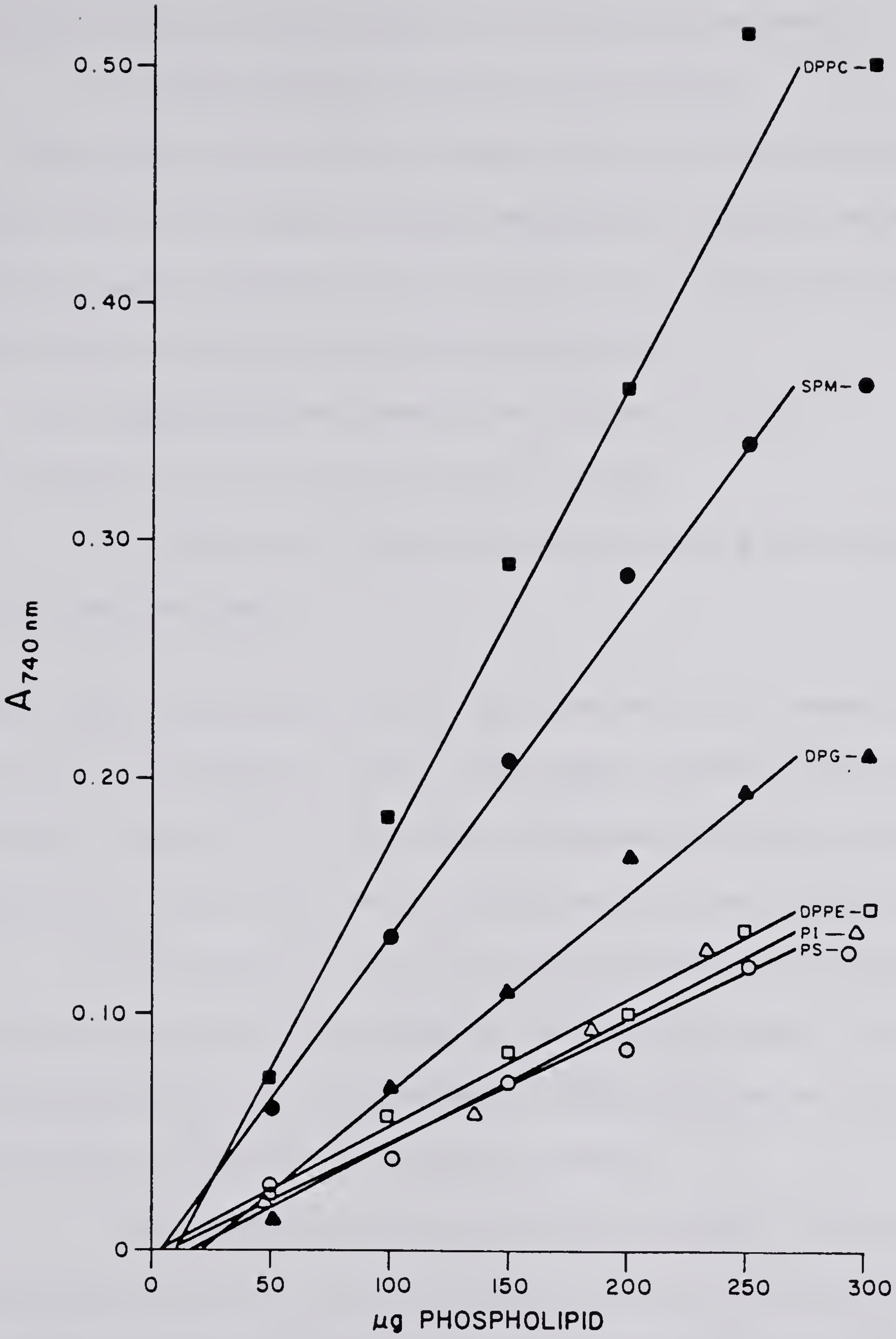


Figure 20. Evaluation of phospholipid standards using the Vaskovsky and Kostetsky spray reagent for color transfer. The standards were DPPC, Spm, DPG, DPPE, PI-crude, and PS using conditions outlined in text.

Based on this initial experiment an attempt was made to study the effect of acid, molybdate concentration, and ANSA concentration on the reduction and transfer of molybdate in order to develop a reagent for phospholipid phase transfer.

The following reagents were used in the evaluation:

(a) Amino naphthol sulphonic acid solution (ANSA) was made up according to Technicon formula AR-24-53. 15 grams of sodium bisulfate and 0.5 gram of sodium sulfate (anhydrous) were added to distilled water and heated to 50° C. Then 0.25 gram ANSA was added to the solution. The solution was mixed and then filtered.

(b) 10% ammonium molybdate solution (AM)—10 grams in 100 ml.

(c) 5N H_2SO_4—13.7 ml concentrated H_2SO_4 in 100 ml.

These three stock solutions were mixed in varying ratios to optimize conditions for reduction and transfer.

Effect of acid concentration on transfer and reduction of color reagent. Solutions containing 1.0 ml of 10% AM and 1.0 ml of ANSA reagent were made up with varying H_2SO_4 concentrations (0.20–4.00 N) by the addition of aliquots of 5N H_2SO_4 and distilled water to a final volume of 10 ml. These solutions were heated 15 minutes in boiling water.

The blue color of the reduced solutions was noted in the samples which contained 0.10, 0.25, and 0.50 N. These three blue solutions were diluted 1:20 and their spectra were measured in the Pye-Unican SP-8-100 UV Spectrophotometer. Figure 21 shows a plot of normality vs. absorbance for the reduced molybdate.

These solutions were then checked for phase transfer. 1.0 ml of acetonitrile: methanol:water (51:35:14 by vol.) was mixed with 0.100 ml of each color solution and 100 μg DSPC (0.020 ml of 5 mg/ml in ethanol). This solution was extracted with 2.0 ml of hexane:toluene (60:40 by vol.). The sample was heated to 50° C for 5 minutes and centrifuged at high speed in a bench top centrifuge for 30 seconds. Figure 22 shows that the optimum acid concentration in the reagent was 0.25 N H_2SO_4 for maximum response at 1% ammonium molybdate.

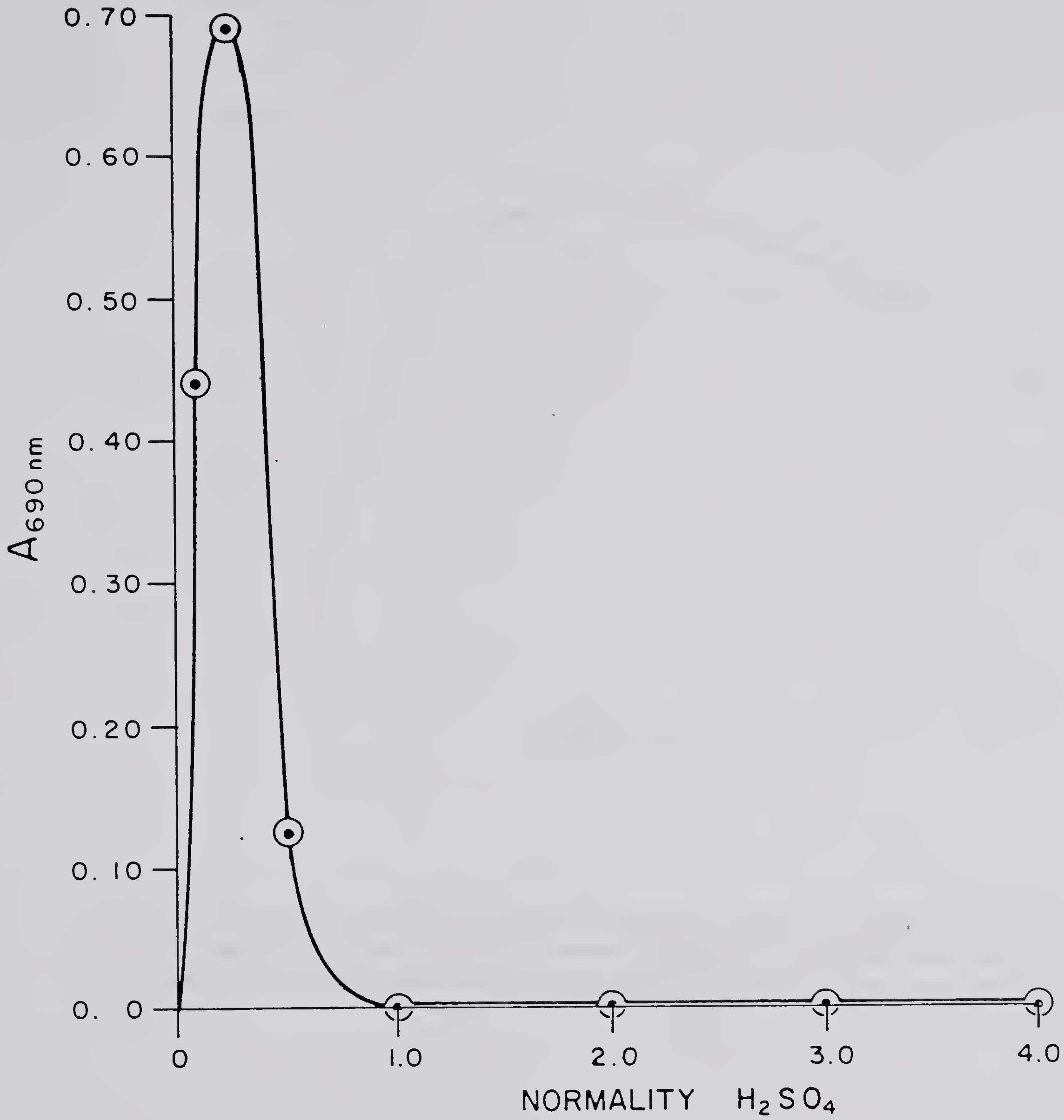


Figure 21. The effect of acid on the reduction of ammonium molybdate by ANSA. Color reagents produced by reduction by ANSA over range of normality using H_2SO_4. The resultant reagent was diluted x20 with distilled water. Conditions outlined in text.

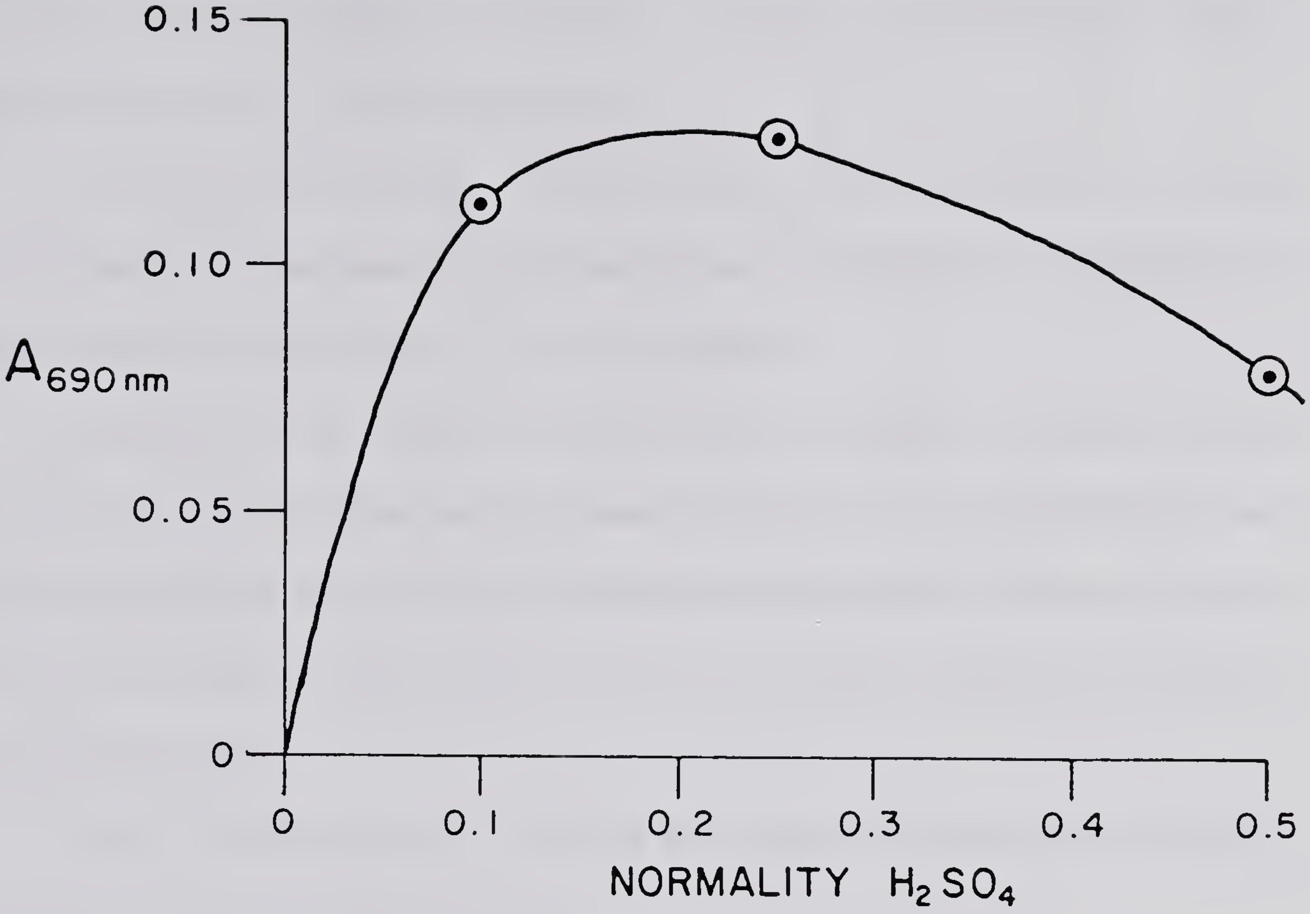


Figure 22. The effect of acidity on the transfer of reduced molybdate by DSPC. 0.100 ml of each color reagent was added to 1 ml HPLC solvent containing 100 μg of DSPC. The resultant color complex was extracted with hexane toluene (60:40 by vol.) and its absorbance was read at 740 nm.

The effect of ammonium molybdate concentration on reduction and transfer. The effect of ammonium molybdate on the reduction and transfer of molybdate-phospholipid complexes was evaluated. A ratio of 1% ammonium molybdate to 0.25 N H_2SO_4 was considered to be optimum from the last experiments. 1.0 ml of ANSA stock was used with the following ratios of AM to H_2SO_4, 1% AM/0.25 N H_2SO_4, 2% AM/0.50 N H_2SO_4, 3% AM/0.75 N H_2SO_4 and 4% AM/1.00 N H_2SO_4.

The solutions were made to a final volume of 10 ml and heated in boiling water for 15 minutes. The absorbance was read at 690 nm of 1:20 dilution in distilled H_2O. The maximum reduction was found in the 2 to 3% AM range.

The solutions were tested for transfer with 250 μg DSPC (0.50 ml of 5mg/ml in ethanol). In 1.0 ml of acetonitrile:methanol:water (51:35:14 by vol.), 0.100 ml of each color reagent was added and mixed. The solutions were extracted with 2 ml hexane:toluene (60:40 by vol.). The solutions were heated at 50° C for 5 minutes, cooled and centrifuged at high speed for 30 seconds.

Figure 23 shows percent molybdate vs. transfer. The solution of maximum transfer occurred at 2% AM and 0.50 N H_2SO_4.

The effect of ANSA concentration on the transfer of colored complex. In the attempt to look at the effect of reducing agent concentration, 2.0 ml AM (10% AM) and 1.0 ml (5 N H_2SO_4) and 0.020, 0.100, 0.500, 1.000 and 2.000 ml of ANSA stock were used. The solution was made up to a final volume of 10 ml and heated 15 minutes in boiling water. The yellow color intensity increased with increasing ANSA prior to heating. During heating the degree of color formation increased with increasing ANSA. Precipitation was noted in the 0.500, 1.000 and 2.000 ml aliquots so these solutions were filtered prior to testing for transfer by phospholipids.

The transfer was checked with 100 μg of DSPC in 1.0 ml acetonitrile:methanol:water (51:35:14 by vol.) by the addition of 0.100 ml of each of the above color reagents. The colored complex was extracted with 2.0 ml of hexane:toluene (60:40 by vol.).

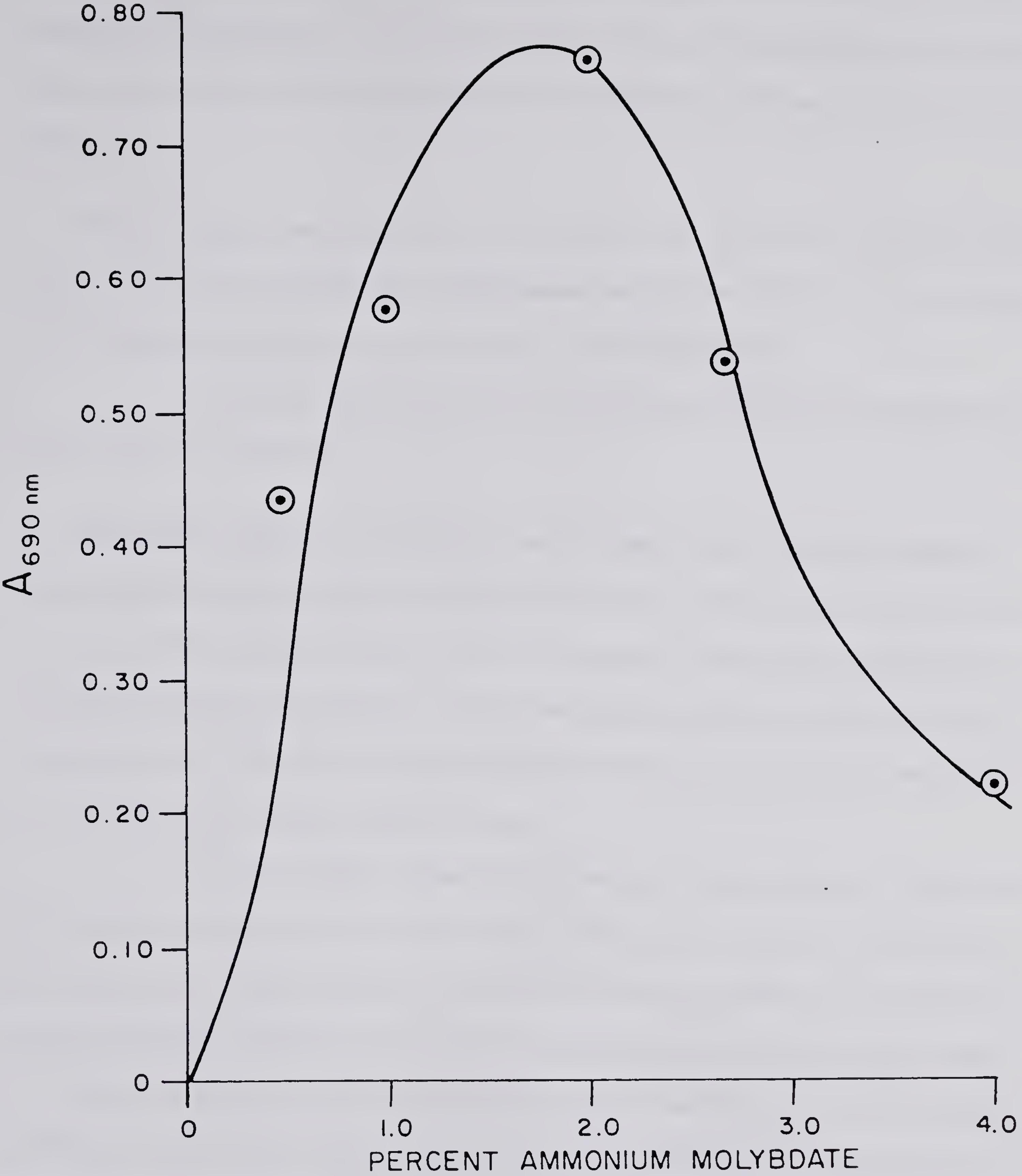


Figure 23. The effect of ammonium molybdate concentration on the transfer of reduced molybdate by DSPC. Conditions outlined in text.

The sample was heated to 50° C for 5 minutes and centrifuged at high speed in a bench top centrifuge. The samples were scanned from 500 to 800 nm. Figure 24 shows that the optimum color extraction was at 0.5 ml ANSA in 10 ml solution containing 2% AM and 0.50 N H_2SO_4.

The stock reagent formulation. Mix 10 ml ANSA stock reagent and 10 grams of AM and 13.7 ml H_2SO_4, add 200 ml of distilled water and dissolve. Make up to 500 ml. Heat for 15 minutes at the high setting on a hot plate, cool and then filter.

Each day a new batch was made, subsequent testing was to be undertaken after evaluation of usefulness.

Automated non-digestive colorimetric analysis using reduced molybdate reagent. A simple automated analysis system was built using a Technicon Autoanalyzer II System coupled to the HPLC separation system, to test the response of soya lecithin phospholipid extract (900 μg injected in ethanol). A top layer extraction method was used as described in Technicon manual TN-0170-01 (111) and the loss of color was measured in the bottom layer by using the inverse setting on the colorimeter.

The color reagent, reduced molybdate stock reagent diluted x 3 (flow rate 0.32 ml/min), was mixed with the HPLC solvent (flow rate 2.1 ml/min). The colored complex formed was extracted with hexane:toluene (60:40 by vol.) (flow rate 0.6 ml/min) by mixing the heating coil at 40° C (20 turns) and removing the top layer using a phase extractor. The loss of absorbance due to phospholipid transfer was measured at a 40% standard calibration on the inverse mode. A simultaneous UV trace was taken for comparison purposes and is seen in Figure 26.

From Figure 25 only one major peak corresponding to PC is noted so it was thought that further studies should be undertaken in the evaluation of reduced molybdate reagent, before returning to development of an automated system.

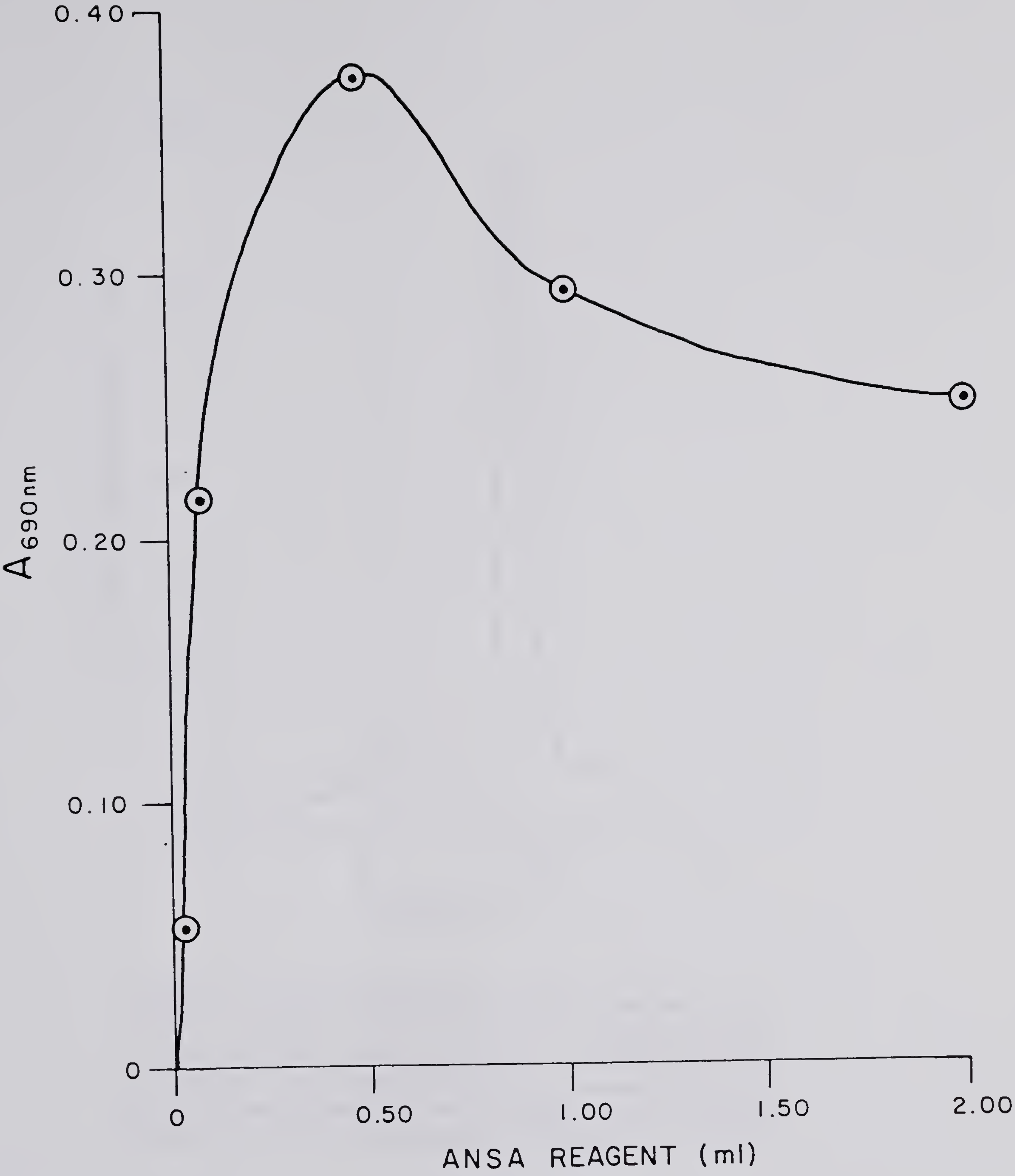


Figure 24. The effect of the reducing agent ANSA concentration on the transfer of reduced molybdate by DSPC. Conditions outlined in text.

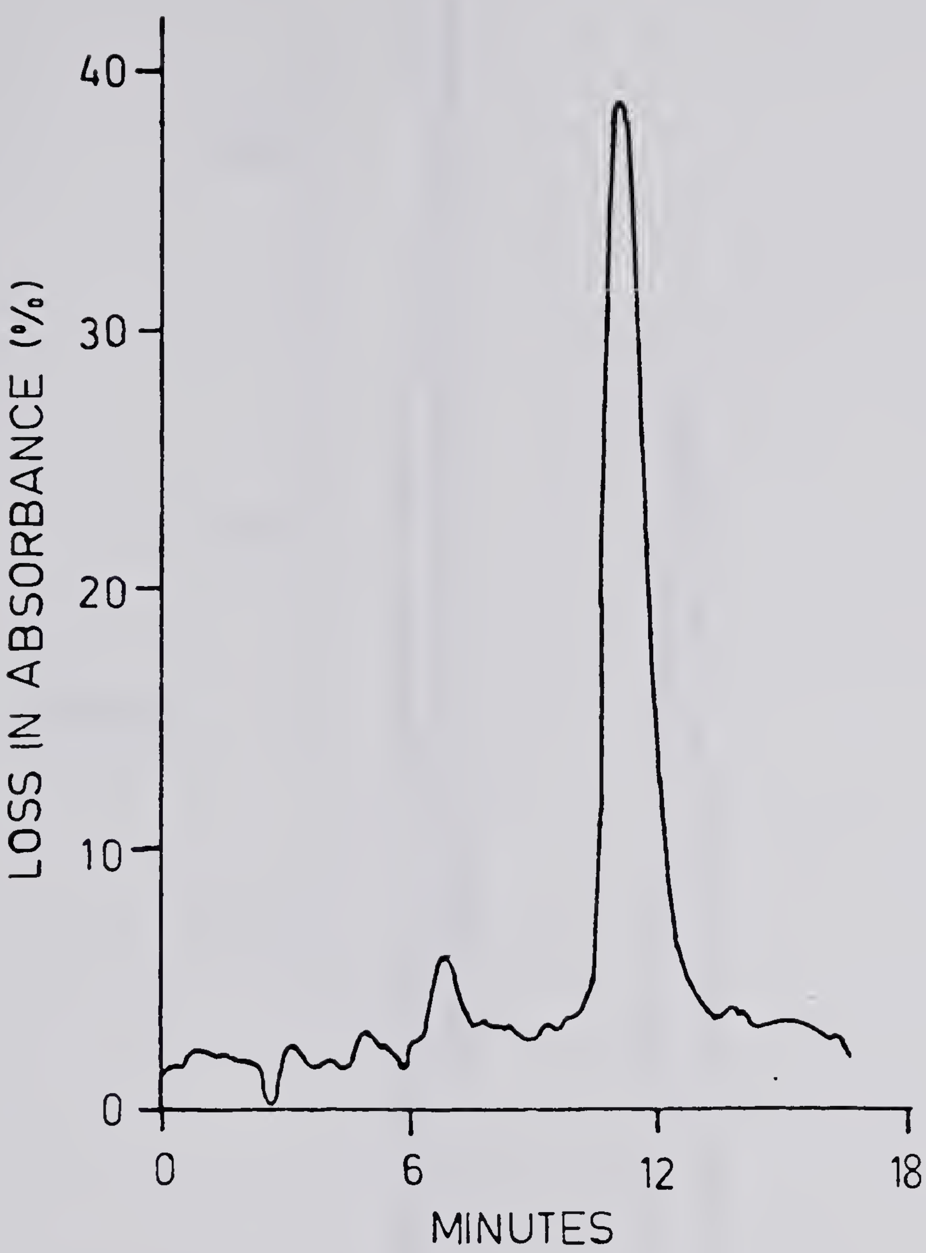


Figure 25. Automated non-digestive colorimetric analysis of soya lecithin phospholipid extract using reduced molybdate. Solvent system, acetonitrile:methanol:water (51:35:14 by vol.); flow rate 2.10 ml/min., room temperature, detection-colorimeter 660 nm cut-off filter, standard calibration 40% on inverse column, Whatman silica gel 10 μ (500 x 4.6 mm). Injection of 900 μg.

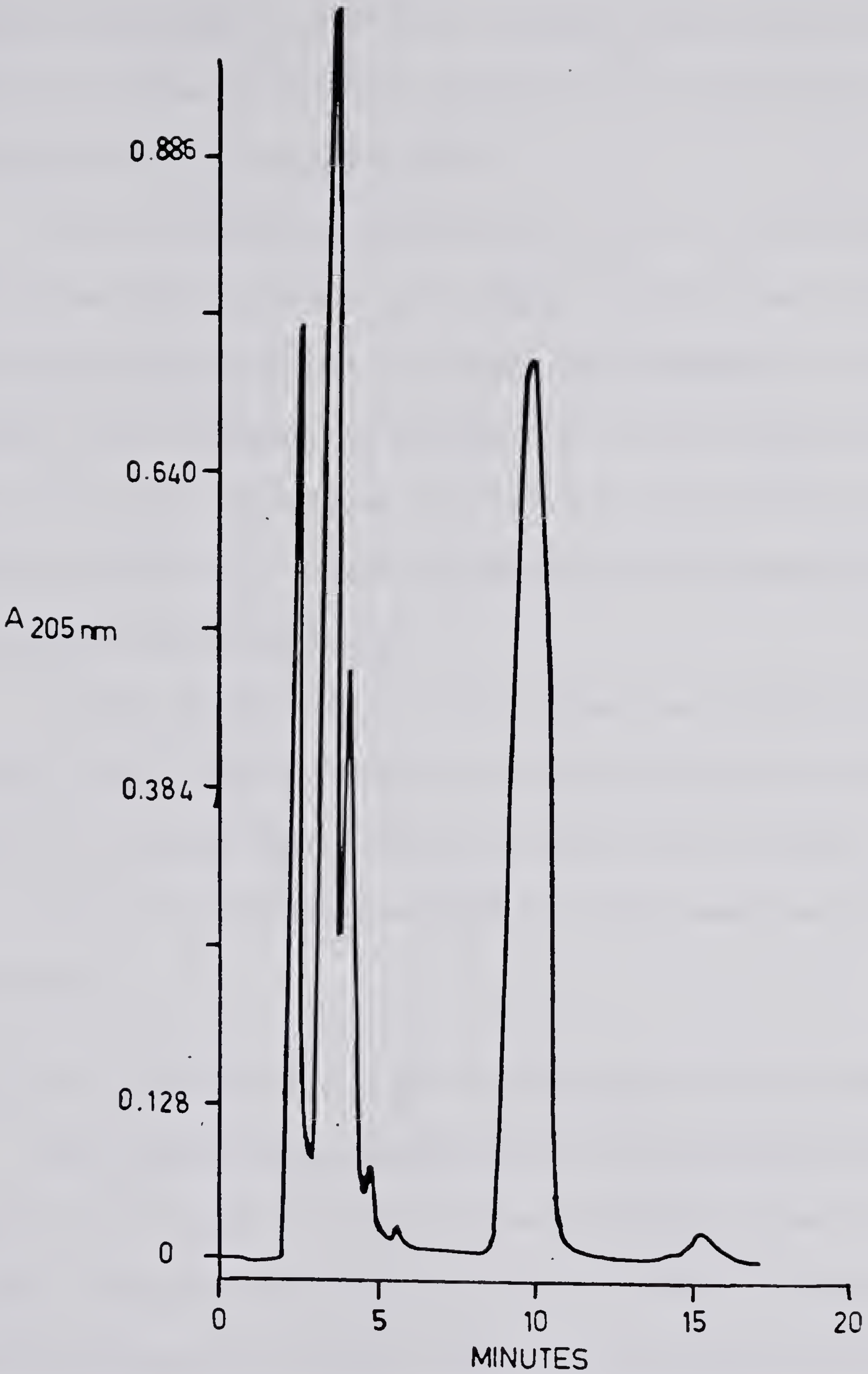


Figure 26. UV detection of soy lecithin phospholipid extract for comparison to non-digestive colorimetric analysis. Ultraviolet monitoring during non-digestive colorimetric analysis (Figure 25) at 205 nm and 1.28 A full scale deflection.

The effect of $HClO_4$ on the transfer of phospholipid ethanolamine. When the stock reagent was used in the Technicon Autoanalyzer system, it was found that PE was not transferred adequately. Subsequently, a quick review of Galanos (88) revealed that the acid content was higher in his system (1.2 N $HClO_4$). The next series of experiments was an attempt to improve conditions by going to a higher acidity.

1.0 ml of acetonitrile:methanol:water (51:35:14 by vol.) containing 100 μg of egg yolk PE and 0.100 ml standard color reagent was tested over the $HClO_4$ range (0.025–0.200 ml concentrated $HClO_4$). The samples were extracted with 2 ml of hexane: toluene (60:40 by vol.). The samples were heated to 50° C for 5 minutes and centrifuged at high speed in the bench top centrifuge. The results are shown in Figure 27. The sample containing 0.100 ml $HClO_4$ was considered optimum for maximum response with minimum acid [equal to acidity used by Galanos (88)].

The effect of color to $HClO_4$ rates on transfer was studied. The samples were treated the same as above except the $HClO_4$ was kept at 0.100 ml and the color reagent was varied from 0.025 to 0.200 ml. Figure 28 shows the results of the experiment.

The 0.100 ml of $HClO_4$ and 0.200 ml of color reagent was used in the subsequent experiments.

Evaluation of PC, PE and Spm through the column using reduced molybdate reagent.

Three phospholipids were studied to see if the colorimetric system using reduced molybdate could be calibrated. The conditions of fraction analysis are those used in previous sections. Varying concentrations (50–500 μg) of DOPC (18:1), Spm (bovine) and PC (egg yolk) were injected into the HPLC system. They were separated on a Whatman silica gel column with acetonitrile:methanol:water (51:35:14 by vol.) at a flow rate of 1.0 ml/min (reduced due to high pressure). 1.0 ml fractions were collected at a rate of one per minute using an ISCO fraction collector. A simultaneous UV trace was taken at 205 nm using Tracor 960 variable wavelength detector (Figure 30).

The peak areas were measured by weighing the peaks and dividing by the

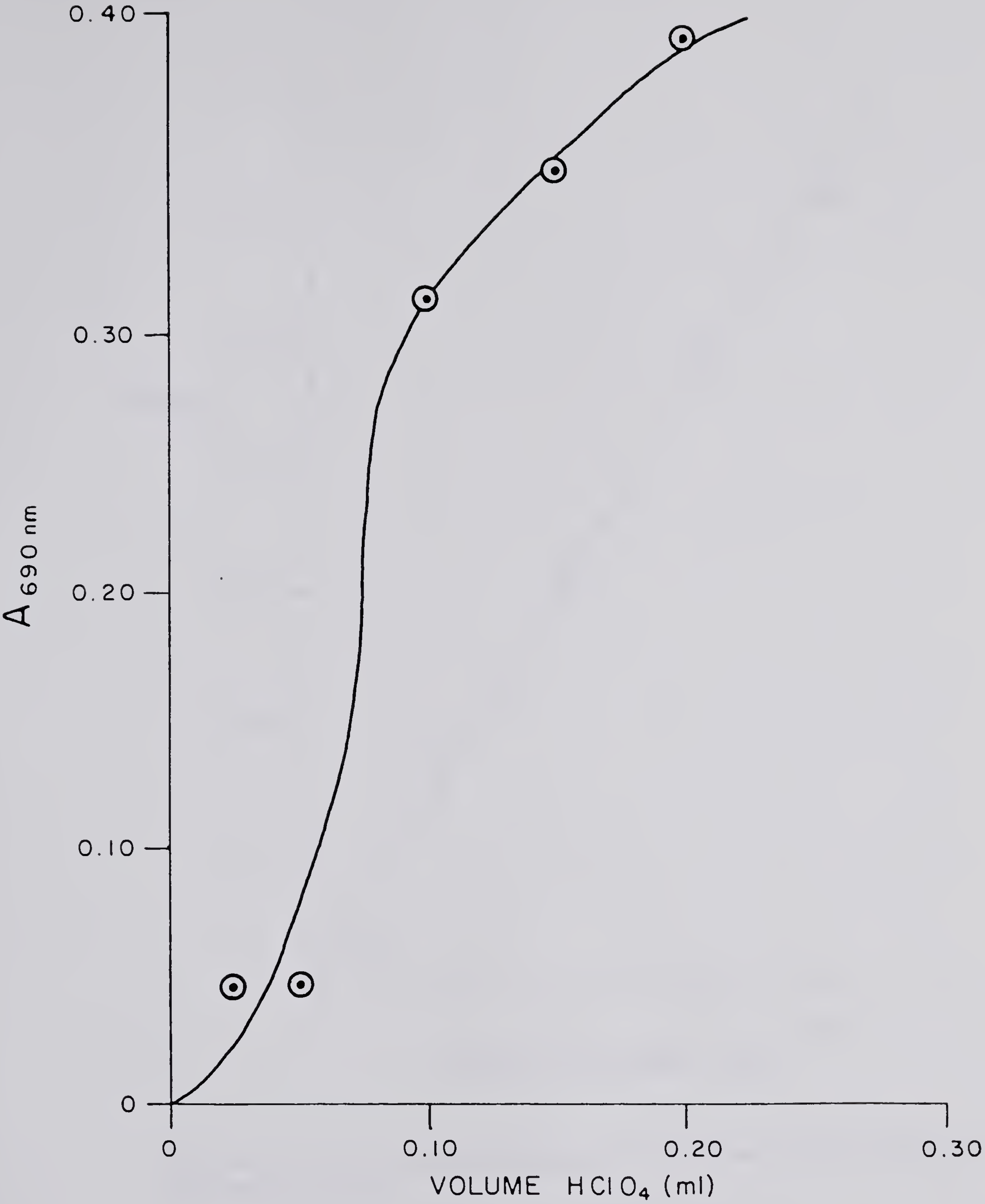


Figure 27. The effect of $HClO_4$ concentration on the transfer of PE. Conditions outlined in text.

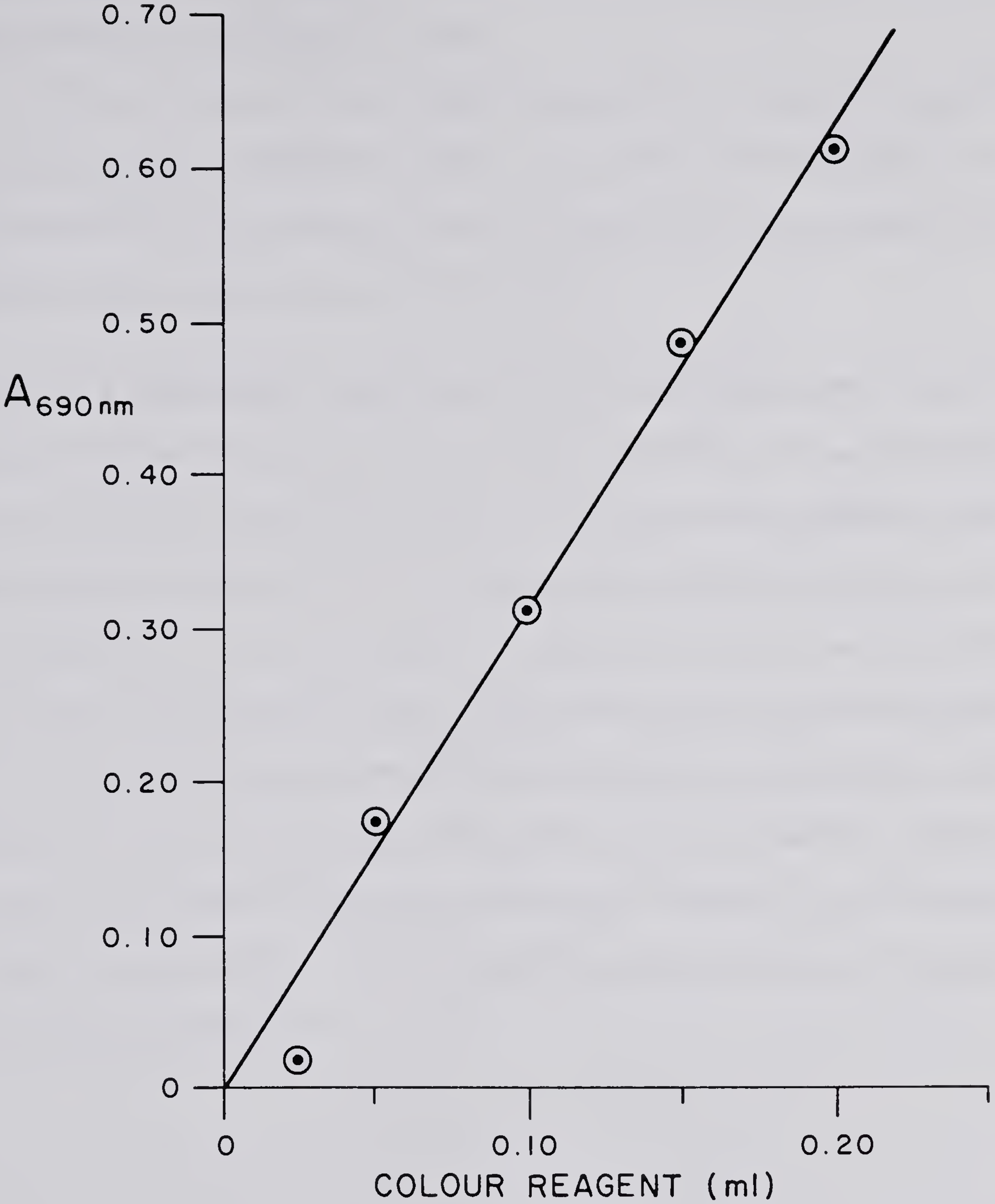


Figure 28. The effect of color reagent concentration on the transfer of PE. The $HClO_4$ was kept constant at 0.10 ml.

weight of a known area (6.15 mg/cm^2 for paper used). The areas were put on the same scale by multiplication of sensitivity (eg., 1.28A full scale deflection area was multiplied by 4) in order to compare to a value on a 0.32A scale.

Figure 29 shows the relationship between peak area measured and μg of PL injected using non-digestive colorimetric analysis or egg yolk PE, DOPC and Spm. An attempt to evaluate DSPC for comparison of fatty acid chain length was unsuccessful due to peak spreading at high concentration (solutibility problem).

Evaluation of a chicken lipid extract using the reduced molybdate system. Because of low response of standards the conditions were slightly modified for the sample analysis. The flow rate of the HPLC solvent was 2.0 ml/min. 1.0 ml fractions were analyzed as earlier but 1.0 ml of hexane:toluene (60:40 by vol.) was used instead of 2.0 ml to increase sensitivity (a microcuvette had to be used). The plot of absorbance vs. fraction number is shown in Figure 31 for a 400 μg injection of chicken breast muscle lipid extract (approximately 210 μg PL) showing good response for PE and PC peaks. The above experimentation is only a preliminary study, further study of the transfer effect has to be undertaken unless the system is used to study effects on one lipid class such as PC. However it is the first non-digestive system of analysis from a column separation requiring a minimum of manipulations and a simple reagent formulation.

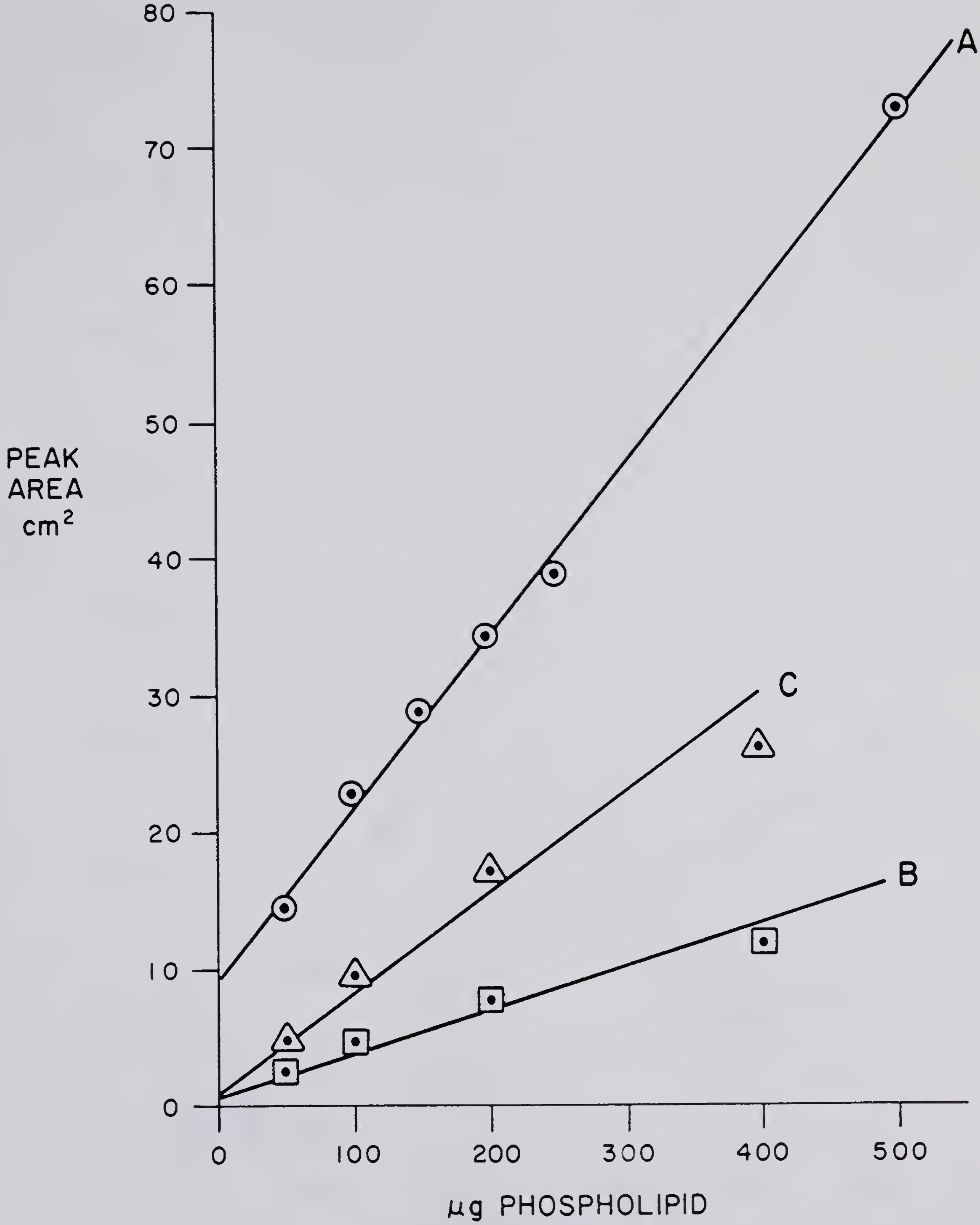


Figure 30. UV detection of phospholipids for comparison to non-digestive colorimetric analysis. Area under curve is shown for A–egg yolk PE, B–DOPC and C–bovine Spm.

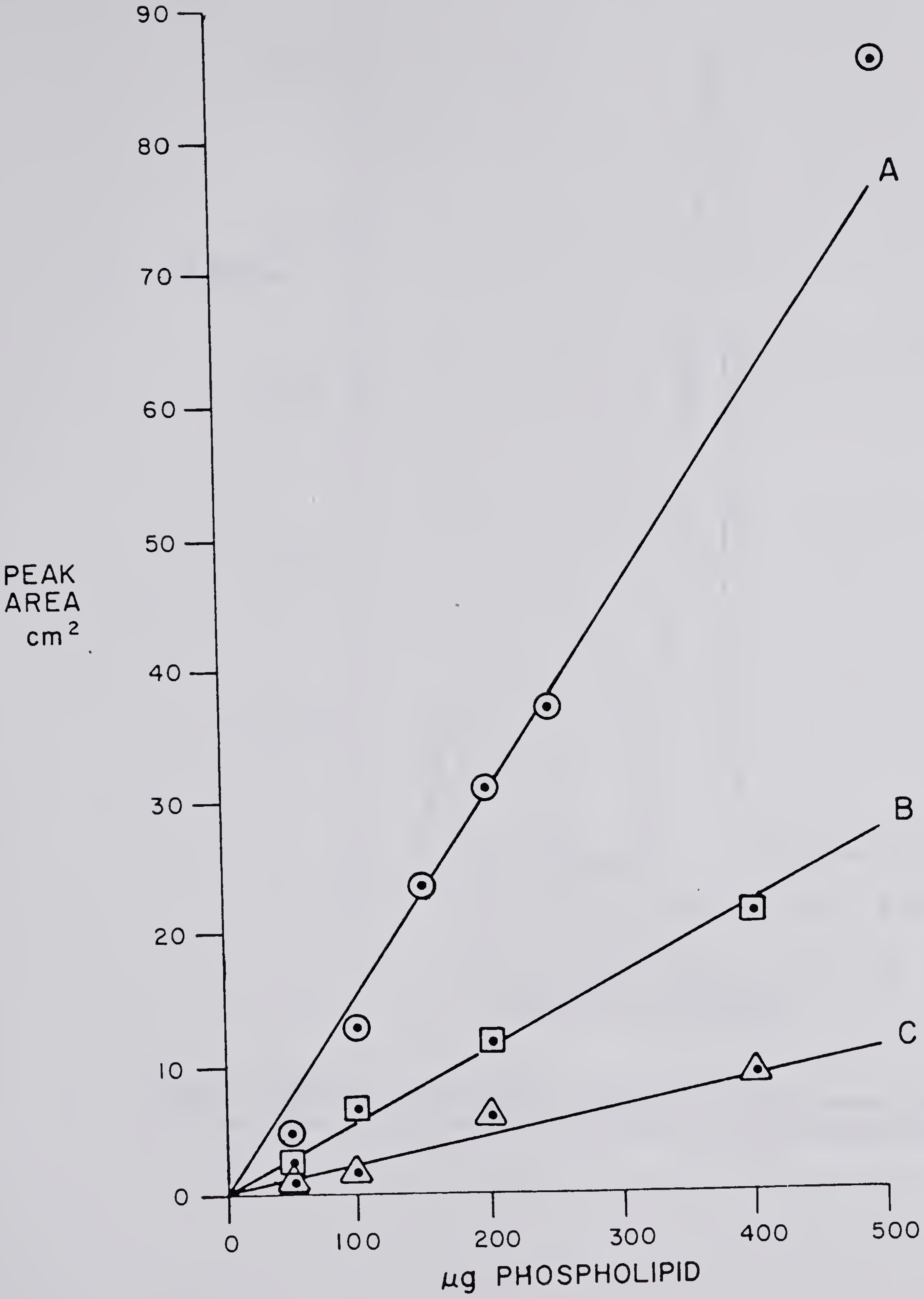


Figure 29. Evaluation of PC, PE and Spm through the column using reduced molybdate reagent. Area under curve is shown for A–egg yolk PE, B–DOPC and C–bovine Spm.

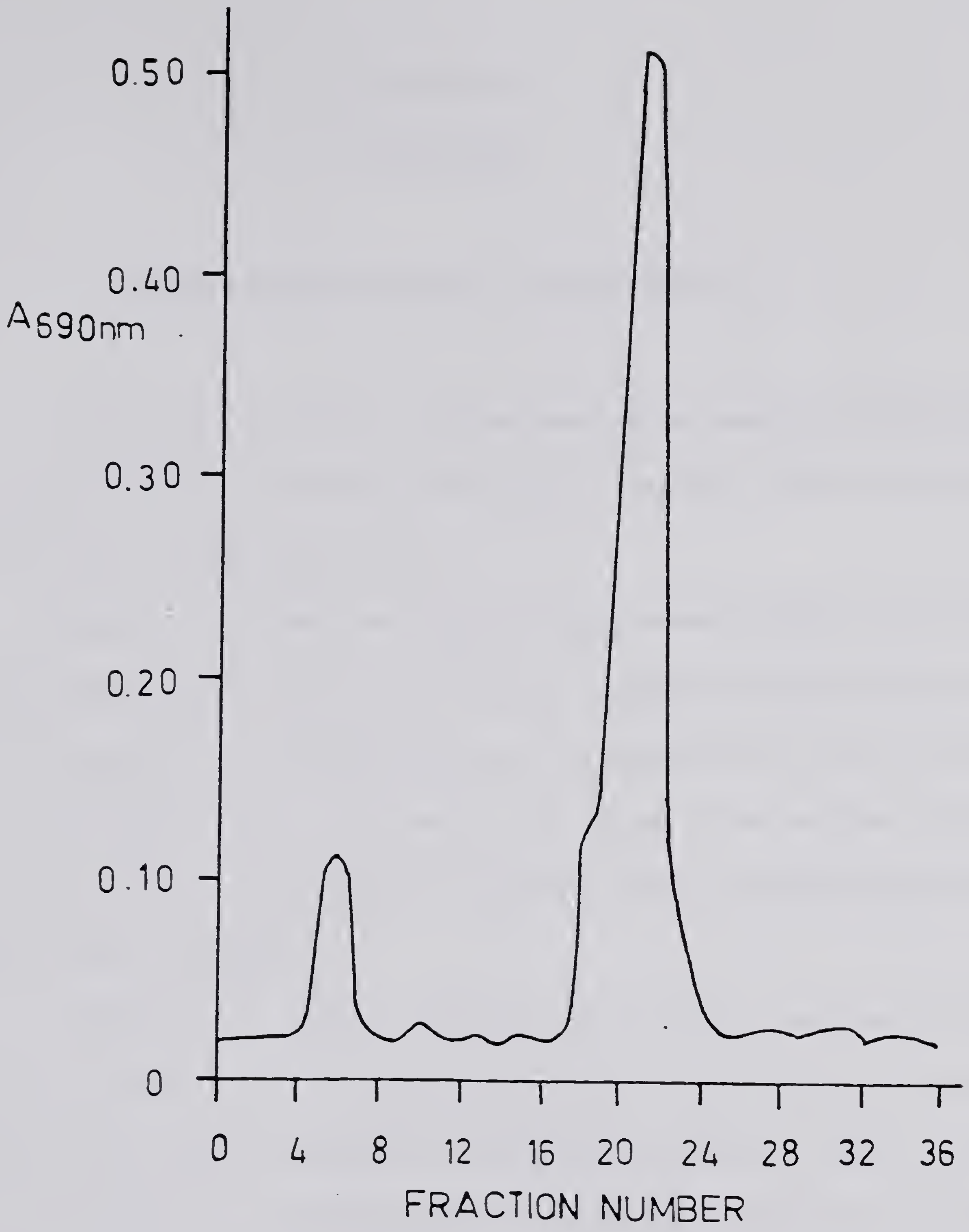


Figure 31. Evaluation of a chicken breast muscle lipid extract using reduced molybdate reagent. Fractions collected at 0.5 min. intervals. Conditions outlined in text.

CHAPTER IV

DISCUSSION

THE HPLC SEPARATION OF PHOSPHOLIPIDS

I found that I was able to separate most of the major phospholipids (PE, LPE, PC, Spm and LPC) into classes using HPLC and UV detection at 205 nm by modifying the solvent system of Jungalwala *et al.* (38).

Preliminary work with the Vydac silica gel column (500 x 2.0 mm) using an acetonitrile:methanol:water solvent system (38) and a hexane:isopropanol:water solvent system (40) for separation of phospholipid standards was unsuccessful. However, the observations of elution behavior made in the above system led me to the conclusion that any further work using another column should be attempted with acetonitrile:methanol:water mixtures under isocratic conditions.

A Whatman silica gel column (500 x 4.6 mm) with a guard column was obtained for further studies. This column was evaluated using a standard mixture of four phospholipids (DPPE, DPPC, Spm and egg-LPC) in several solvent systems. I found when using 14% water and adjusting the acetonitrile:methanol ratio that the retention times increased with increasing acetonitrile (see Table 3). From the above evaluation I concluded that the acetonitrile:methanol:water (51:35:14 by vol.) solvent mixture was an adequate system to be used in further studies because it gave maximum resolution from the solvent front in minimum time. In this solvent system I was able to separate PE, LPE, PC, Spm and LPC, but the standards, PI and DPG were found to elute at the solvent front. PS was found to elute close to the PE peak and may interfere with PE resolution from the solvent front. In the meat samples studied, PS is only a minor constituent as compared to PE so the interfer-

ence would be minimal.

The above solvent system was applied to the separation of several natural phospholipid mixtures into classes. Figure 9 is an example of beef muscle phospholipid extract separation. I compared the peaks of this separation with elution of natural phospholipid standards and found good retention time agreement between sample and standards for PE, LPE, PC and Spm. Sample LPC had a longer retention time than the standard LPC possibly due to a difference in fatty acid chains present on each of the LPCs. I also noted in the beef muscle extract an unknown peak at 18.5 minutes, but was unable to identify it using the standards I had available.

From the analysis of natural lipid extracts I found it was not necessary to separate the neutral lipids from the polar lipids prior to injection, which is usually necessary prior to TLC analysis. The neutral lipids are eluted at the solvent front, but if the quantity of neutral lipids is too large it may be necessary to use only a polar lipid extract to prevent clogging of the column and masking of PE and LPE peaks by a large solvent front. The use of an absorbing injection solvent caused a large solvent front peak which also interferes with PE and LPE peaks. This is prevented by dissolving the sample in a low absorbing solvent such as high purity ethanol (98%) or by diluting a concentrated extract by 50 or more in the HPLC eluent to minimize the absorption of the extraction solvent.

In order to see if it was possible to calibrate the UV detection of phospholipids, I studied four PC and three LPC standards. I was able to see differences in UV response and retention times.

The variation in retention time can be seen from Table 5 and Figures 10 and 11. The saturates had longer retention times for PC. Among the LPCs, 18:2 had a longer retention time than 18:1 LPC which does not agree with the pattern found among the PCs where 18:2 elutes before 18:1 PC. The differences of retention times found here do not seem to affect resolution of peaks in natural standards and account for the differences in retention times mentioned earlier between standard LPC and that of a meat sample.

The absorption of the standards seems to be dependent on the number of double bonds present in the fatty acid side chain. I compared DPPE (16:0) and DPPC (16:0) and found their UV responses to be similar for the same quantity of lipids. When the side chain was increased to 18:0, the PC had an increased absorption. The 18:2 PC had approximately 12 times the absorption of 18:1 PC which may be due to peroxidation of the double bond which could lead to conjugation of the double bonds in the 18:2 fatty acids. Conjugation of double bonds is known to enhance absorbance. It was noted that 18:2 LPC had only 25% of the absorbance of 18:2 PC (for equal concentrations) so some as yet unidentified effect must enhance the absorbance due to the double bonds present. The natural LPC standard from phospholipase A action on egg lecithin had similar absorbance to 18:1 LPC but they were both about one-third of 18:2 LPC.

During other studies I noted a difference in Spm from different sources and thus egg yolk PE had a substantially higher absorption than DPPE probably due to the fact that natural phospholipids contain a higher number of unsaturates. I also found that the UV response was linear for egg yolk PE, Spm and DOPE, as seen in Figure 3, but these curves could not be applied to an unknown sample because the extinction coefficients would be different. This is probably due to the sample having a different mixture of fatty acids in each individual class.

From the information outlined above I concluded that HPLC is useful for the separation of phospholipids but UV detection cannot be used for quantification unless the fatty acid composition is known for each class in a sample phospholipid mixture. This same conclusion has been reached by two other workers (38, 40).

I concluded that in order to make the HPLC a useful analytical tool in phospholipid analysis an alternative method of quantification has to be found which is not as dependent on fatty acid chain composition. This is the justification for the study of non-digestive techniques with subsequant automation.

THE DEVELOPMENT OF NON-DIGESTIVE TECHNIQUES FOR THE MEASUREMENT OF PHOSPHOLIPIDS IN THE HPLC ELUENT

Introduction

Digestive techniques can be used to analyze phospholipid phosphorus in the HPLC eluent but because of the manipulations and special precautions required the time advantages of rapid separation are lost. The malachite green method of Bowyer and King (71) is adequate for the measurement of phosphorus in phospholipids separated by HPLC as shown in Figure 14, but it is not suitable for routine analysis. The problems inherent in the use of UV detectors have been discussed in the first portion of this thesis. The use of the moving wire transport FID involves the same problems as the UV detector because the response is dependent on the carbon chain length in the fatty acids of the phospholipids as outlined by Kiuchi *et al.* (49). If a simple method could be found to quantify phospholipids in the eluent, possibly by colorimetric means, HPLC could become a useful tool for the lipid chemist.

Color Transfer Using Vaskovsky and Kostetsky Spray Reagent

The widely used non-digestive technique of Raheja *et al.* (89) (cited 63 times in *Science Citation Index* in the period 1973–1977), for the analysis of phospholipids was studied for possible application to quantification after HPLC separation. Work with this system led to the development of a second reagent for non-digestive measurement of phospholipids.

Raheja *et al.* (89) stated that the mechanism of the assay is the direct reaction of the chromogenic solution with the phospholipid phosphorus, which leads to the formation of a Prussian blue complex. The complex is separated from background reagent color by solubilization in chloroform. Sundhu (91) described the same technique as “color development” with intact phospholipids.

Based on the information outlined in these papers I set out to measure phospholipids in the HPLC eluent using the same chromogenic reagent, the Vaskovsky and

Kostetsky spray reagent (95).

A sequence of observations led me to conclusions which greatly simplified the application of this reagent to my HPLC system. Table VI outlines the conditions which were used to detect phospholipids in the HPLC eluent. The mechanism is not understood, but it was implied by the first workers (89, 91) that a "reaction" of phospholipids with the chromogenic reagent is required for color development. The first experiment was to determine if the phospholipid reaction with chromogenic reagent could be differentiated from the excess reagent in the HPLC eluent. This was carried out with heating but it was not possible to differentiate the reacted phospholipid reagent color from the excess reagent color. I concluded that the mechanism must be based on extraction of a colored complex of chromogenic reagent and phospholipids. This experiment is reported as condition 1 in Table VI.

Based on this observation a lipophilic extracting solvent was sought. The extracting solvent was required to be immiscible with the HPLC solvent to extract the phospholipid-chromogenic reagent complex quantitatively and not to extract any uncomplexed reagent. Among the solvents studied heptane, hexane, isopentane, and pentane were found to have excellent phase separation qualities.

Heptane was the first extractant used to remove the phospholipid color reagent complex from the HPLC eluent. The transfer was linear up to 1500 μg, but at this point precipitation of the blue complex was noted. In this study the samples were heated as in the Raheja *et al.* (89) method. The precipitation showed that the colored complex had a maximum solubility in heptane, revealing one of the first limits on the design of the system. This was the second condition reported in Table VI showing it is possible to measure the phospholipids in the HPLC solvent.

Hexane was adapted as the extractant because it is more widely used as a solvent in lipid chemistry (110). I studied the extraction of the colored complex with hexane under four conditions (conditions 3–6, Table VI) which led to a simplified method

Table VI

Conditions of Phospholipid Detection Using the Vaskovsky and Kostetsky Spray Reagent

No.	Vol. of Reagent	Vol. of Solvent	Color of Solution after Addition of Reagent		Treatment of Reagent Prior to Use	Heating of Sample Mixture	Extractant Used	Color Detection in Extractant	Conclusion
			no PL	PL					
1	0.100	1.00	blue	blue	no treatment	+	not used	-	Cannot differentiate any color change with PL present without extraction
2	0.300	1.50	green	slightly blue	no treatment	+	heptane	+	Extractant required
3	0.100	1.00	blue	blue	no treatment	-	hexane	+	Heating not necessary
4	0.150	1.00	green	slightly blue	no treatment	-	hexane	+	Lipid can turn the solution slightly blue by itself
5	0.150	1.00	green	slightly blue	heating of color reagent prior to use	-	hexane	+	Heating will not change color reagent color
6	0.150	1.00	blue	blue	air bubbled through prior to use	-	hexane	+	Air turns the color reagent permanently blue

for application of non-digestive techniques to the measurement of phospholipids in the HPLC eluent.

In condition 3, I added 0.100 ml chromogenic reagent and the HPLC solvent turned blue. I was able to extract the phospholipid-chromogenic reagent complex and concluded heating was not necessary. In condition 4, I added 0.150 ml and found the solvent did not turn blue possibly due to the acidity of the color reagent which would stabilize the green color of the chromogenic reagent. Another observation was that the presence of phospholipids with the chromogenic reagent caused the formation of a blue colored complex which is extractable. Attempts to measure the blue color complex formation were unsuccessful because the complex precipitated out in the HPLC solvent causing turbidity.

In condition 3, if the amount of chromogenic reagent was increased, the blue color reverted back to green possibly due to an acidity effect. Whether the solution was green or blue, color transfer still took place, though transfer was higher with 0.150 ml color reagent.

In condition 5, I found that heating the color reagent alone did not change it to a blue state. In condition 6, air bubbled through the color reagent caused it to turn permanently blue (irreversible upon addition of more color reagent). A complex could be formed which was transferable using this blue reagent. This blue solution is possibly a blue oxide of molybdate as described by Cotton and Wilkinson (112) formed by the interaction of O_2 with the chromogenic reagent. The reagent produced in condition 6 was later found to be useful in automated systems because the measurement of loss of color due to quantitative phospholipid transfer of color into the organic layer could be used.

From the above observations I concluded that the possible mechanism of the reaction is the formation of an association complex of the phospholipid with the color reagent which is then transferred into the organic layer. The molybdate complex can take a number of forms but Mo^{V} must be present for detection. [Galanos (88) showed Mo^{VI} can also be transferred. I was also able to transfer Mo^{VI} and reduce it to Mo^{V} after extraction,

still producing a blue colored complex). I found heating was not necessary for complexing or color development of the extracted complex as was considered the case by other researchers (81, 91).

From the observations made above, a simple system was developed where the color reagent is added to the HPLC eluent containing the phospholipids, mixed and extracted with hexane. In this system DPPC was tested using 0.150 ml color reagent (not oxidized) and 2 ml of hexane as the extractant. The response was linear from 25 to 250 μg and it was higher than when heptane was used as the extractant.

Because of the expense of mercury in the reagent, the effect of mercury concentration was examined but at the time of the experiment the function of mercury was unknown. I wanted to decrease the concentration of mercury to make a less expensive reagent for use in automation. It was found that 2.5 ml of mercury in the formulation gave a slightly higher response than the 5.0 ml. But since mercury is the reducing agent in the mixture as stated by Lucena-Conde and Pratt (99), it should be possible to maximize the reduction of Mo^{VI} to Mo^{V} by allowing it to be reduced overnight, if mercury did not cause further reduction from the Mo^{V} to Mo^{IV} state. For all subsequent tests the mercury was halved (2.5 ml Hg) in the formulation. The responses reported in Figure 17 could only be explained by the fact that the extent of the reduction may be critical in complex formation. The amount of reduction that takes place may be critical to the type of association complex formed.

Using hexane and the formation of the color reagent outlined above, Spm, LPC, DPG, PS, DPPE, LPE and PI were checked for transfer. Spm gave good transfer; likewise DPPC, (LPC caused interfacial changes between phases) but the remainder of the phospholipids showed no transfer.

Toluene was examined and was shown to transfer DPPE but turbidity was present possibly due to the water in the system. Hexane did not transfer DPPE but had excellent transfer qualities for Spm and PC. Mixtures of hexane and toluene were evaluated

by eye for transfer and turbidity. At 60% hexane and 40% toluene, no turbidity was present and transfer of the colored complex still took place for DPPE. This solvent mixture was used in all subsequent studies. Pure toluene can be used if the sample is centrifuged but considering adaptation to automation, it is more desirable to have no turbidity and rapid separation of phases even though the amount of transfer seems lower.

The above extractant was used to test the effect of color concentration on extraction of the colored complex. The optimum was found to be 0.150 ml in 1 ml of solvent. This is approximately 3N H_2SO_4. In the next experiment the effect of H_2SO_4 on the transfer was tested but the color reagent volume was decreased to 0.100 ml. It was found that the maximum transfer was at 0.40 ml concentrated H_2SO_4. The total amount of acid in the color reagent and the added acid equalled approximately 3.3N. This shows that acidity is probably more critical to transfer than the reduced molybdate in the color reagent, as long as the molybdate is in excess of the lipid. Cotton and Wilkinson (112) state that the nature of complexes with Mo^V species is dependent on the anions present and on the pH and the concentration of Mo^V. NaCl had little effect on the color transfer between 0.01 and 0.1 M, hence no salting out effects which might improve transfer occurred. To simplify the procedure no salt or acid was added, only 0.150 ml of color reagent was used in subsequent analysis.

To obtain reproducible results it was found that vortexing had to be timed. A heating of 5 minutes at 50° C improved transfer and centrifugation eliminated any turbidity decreasing control absorbance to almost negligible values. In an automated system the mixing would not be variable and any turbidity would give a constant background.

Under the conditions outlined above, DPPE, DPPC, LPC, DPG, PI, and PS [using 0.150 ml color reagent and 3 ml hexane:toluene (60:40 by vol.] in 1 ml HPLC solvent) were measured for transfer. DPPC had the highest response with Spm, at 75% of DPPC. DPG was much lower, but DPPE, PI, and PS all had less transfer, possibly due to their solubility characteristics.

Differences in polarity between the phospholipids could account for differences in the transfer response of their complexes. Raheja *et al.* (89) possibly overcame this problem by minimizing the aqueous phase of their system and using a large volume of extractant which would maximize both transfer and solubility. In my system I am partitioning the phospholipids between an acetonitrile:methanol:water mixture and a hexane: toluene extractant. It is possible that in the case of the acidic lipids only a portion of the total lipids was transferred from the HPLC solvent to the extractant. Raheja *et al.* (89) used an aqueous phase which was very acidic and small in volume which would facilitate transfer. They were able to transfer LPC; I could not transfer LPC or LPE in the system outlined above.

One of the great advantages of my system is that the lipid is measured directly in the HPLC eluent so no evaporation step is required, but this puts a limitation on the response for acidic phospholipids. Though the transfer may not be complete for all phospholipids as long as it can be linearly calibrated, low responses may be tolerated.

Color Transfer Using the Reduced Molybdate Reagent

The cost and toxicity of mercury make it prohibitive as a reducing agent for the molybdate reduction. Upon a study of the literature [particularly from the information presented by Galanos (88)], I interpreted that reduced molybdate is possibly one of the species being transferred by the phospholipids. From my earlier work with the Vaskovsky and Kostetsky spray reagent (95) I found that a molybdenum blue form of the reagent was transferable without heating. Therefore I wanted to produce a simple molybdenum reagent in the blue form that would be quantitatively transferred by phospholipids without using mercury as a reducing agent.

In Galanos' (88) work a complex was formed by heating AM, ANSA, and the phospholipids together and then extracting into chloroform:ethanol (4:1 by vol.). I have found that is is not necessary to have the phospholipids present during reduction (or molybdenum blue formation), the complex can be directly transferred in its blue state into the or-

ganic layer.

Based on the above observation I attempted to produce a molybdenum blue reagent (or a reduced molybdate reagent) which does not require any mercury and is changed to the blue state prior to use by another reducing agent such as ANSA.

Using AM, ANSA and H_2SO_4 I tried to develop and optimize a reduced molybdate reagent for the non-digestive measurement of phospholipids. Firstly, I evaluated acid effects on both reduction of AM and transfer of a phospholipid reduced molybdate complex. I found that the maximum reduction was at 0.25N H_2SO_4 which incidently, corresponds to the acidity of maximum transfer of the colored complex in this experiment. In the next experiment the ratio of H_2SO_4 to AM was kept constant and the effect of increasing molybdate and acid was simultaneously examined. Two percent AM and 0.5N H_2SO_4 gave optimum transfer in this experiment. This was also the point of maximum reduction in the color solution.

The effect of concentration of the reducing agent (ANSA) on transfer of the phospholipid-molybdate complex was studied and 0.5 ml in a 10 ml formulation was found to be optimum (formulation is outlined in the "Results" section). From the above experiments a stock formulation was developed containing 2% AM 0.5N H_2SO_4 and 5% stock ANSA.

During the evaluation of the above reagent PE was found not to transfer color but if the acidity was increased using $HClO_4$, transfer of color was possible. A 0.100 ml addition of $HClO_4$ was used to increase transfer of PE. I also found that the addition of the $HClO_4$ prior to addition of the color reagent gave better transfer, but the reasons for this are obscure.

When the acid was kept constant at 0.100 ml and the amount of color reagent was increased there was a linear response directly proportional to the color reagent concentration, but at one constant color concentration phospholipids gave a linear response (see Figure 29).

Since the objective was to measure phospholipids after HPLC separation in the eluent, calibration curves were produced by collecting fractions after column separation

of standards. During this study samples were monitored by UV detection and fractions were collected to be analyzed by non-digestive colorimetric analysis. The response was linear and extremely high for egg yolk PE as compared to PC and Spm (see Figure 29). This does not correlate well with the response found with the spray reagent, possibly due to differences in the complex formation and the solubility of the standards used. (Egg yolk PE has a higher solubility than DPPE in hexane.)

In this system I attempted to evaluate the effect of fatty acid chain length using DSPC but its chromatographic behavior was different at high concentration and could not be quantified through the column using this method. In another experiment comparing fatty acids (directly in test tube without column separation) I found that dilinolenoyl PC (18:3) and dilinoleoyl PC (18:2) gave lower transfer than dioleoyl PC (18:1) possibly due to changes in solubility characteristics induced by peroxidation.

DOPC (18:1) and DPPC (16:0) were compared over a range of 20 to 200 μg. It was found that there was a 33% increase in color transfer of DPPC over DOPC. During the above and subsequent analyses in the test tube, a non-linear response was noted below 20 μg phospholipid, possibly due to a very slight solubility of phospholipid-molybdate complex in the HPLC solvent. This makes concentrations below 20 μg undetectable but refinements in solvent and extractant may help overcome this problem.

In order to learn if a natural phospholipid mixture could be analyzed by this method, a chicken muscle extract was subjected to HPLC separation with subsequent non-digestive analyses and the response is shown in Figure 31. Two peaks can be noted: one for PE and a second for PC but the minor components cannot be quantified possibly due to the detection limit of the color reagent (20 μg PL).

The Complex Nature of Molybdenum Reagent-Phospholipid Compounds

Throughout the discussion, transfer of color has been referred to many times. The color being transferred, regardless of the reagent used, could be described as "molybdenum blue" [Raheja *et al.* (89) described it as a Prussian blue complex formation].

Unfortunately, the exact structure of molybdenum blue is unknown, but Cotton and Wilkinson (112) state that "The compounds in which the mean oxidation state of Mo is between the 5 and 6 are the blue ones, e.g., $MoO_{2.0}(OH)$ and $MoO_{2.5}(OH)_{0.5}$." It is beyond the scope of this thesis to determine the exact nature of the complex being transferred; but one thing that can be said is that molybdenum in the Mo^V state is necessary in the reagent for color detection of the molybdenum blue-phospholipid association complex.

Probably the molybdenum blue-phospholipid complex formed by the Vaskovsky and Kostetsky spray reagent (95) is different from the one formed when molybdate is reduced by ANSA, as indicated by the fact that their absorption maxima are different. The association complex with DOPC for the spray reagent exhibits a maximum of absorption at 740 nm while the absorption maximum is at 690 nm for the ANSA reduced molybdate extracted using hexane:toluene (60:40 by vol.).

In both systems the transfer seems to be dependent on pH, color reagent concentration, temperature, mixing and extracting solvent.

PRELIMINARY INVESTIGATIONS OF AUTOMATION USING A SOLVENT EXTRACTION METHOD

During all the experiments on the development of non-digestive techniques for application to the quantification of phospholipids after HPLC separation, the desirability of automation has been kept in mind. I have attempted three automated systems during the course of this work, obtaining partial success which has influenced further experiments in the off-line methods.

To outline the details of each system would not be fruitful at this point in time, because more development work is required, but the positive aspects of each method and their limitations would help to clarify their potential.

The automation was carried out employing the Technicon II Autoanalyzer system using a colorimeter with a 660 nm cut off filter, and in some cases a water bath set

at 40° C.

System I was based on the initial experiments using hexane as an extractant. The observation that bubbling air through the Vaskovsky and Kostetsky spray reagent (95) turned it permanently blue, enabled the loss of blue color in the bottom layer to be measured rather than the complex transfer to the top layer. I was also able to introduce hexane through the air bar eliminating the need for air bubbles in the system. I found it was not necessary to use a phase extractor because the hexane solvent units were removed by the debubbler in the colorimeter. The system gave good response for PC and Spm (DPPC was linear in the range 10–80 μg). DPPE, DPG, PE and PS gave no response.

Solvoflex tubing was used throughout and the system was very stable and could have the potential for the measurement of PC and Spm in such important materials as amniotic fluid. Figure 32 shows the separation and quantification of beef muscle lipids analyzed by this sytem.

In System II the hexane extractant was replaced by benzene. Benzene was chosen because it transferred all the phospholipids mentioned above and turbidity was not a problem, since the bottom layer was measured. Benzene extraction required a phase extractor and it was noted that transfer increased with increasing acidity. PE, PS, PI, DPG, PC and Spm gave a response in the automated system (DPPC was linear in the range 50 to 250 μg). The benzene system worked very efficiently for a short period of time before the analysis system went out of control because of dissolution of the tubing. This was a problem even when the specialized Acidiflex tubing was used. But the positive results of this system led to the conclusion that it is possible to study the major phospholipids using automated non-digestive techniques. However either a less corrosive color reagent or a more resistant tubing is required. The analysis of a soya lecithin phospholipid extract is shown in Figure 33.

System III using the reduced molybdate reagent is outlined in the "Results" section. The experience gained from System I and II permitted the rapid assembly of a

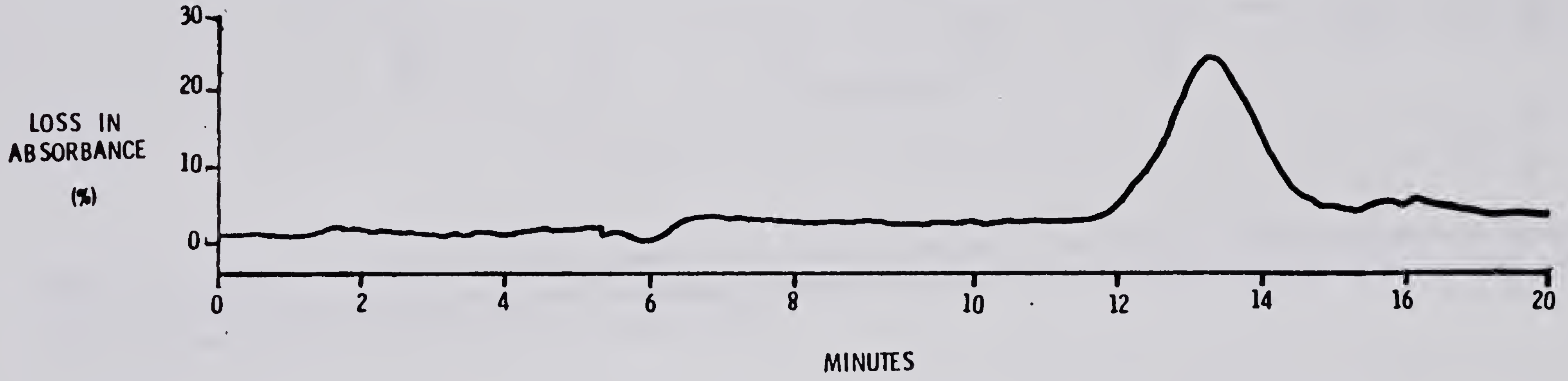


Figure 32. Automated non-digestive colorimetric analysis of beef phospholipid extract using System I. Solvent system; acetonitrile:methanol: water (51:35:14 by vol.); flow rate 2.20 ml/min., room temperature, detection colorimeter 660 nm cutoff filter, standard calibration 10% on inverse, column; Whatman silica gel 10 μ(500 x 4.6 mm). Injection of 200 μg.

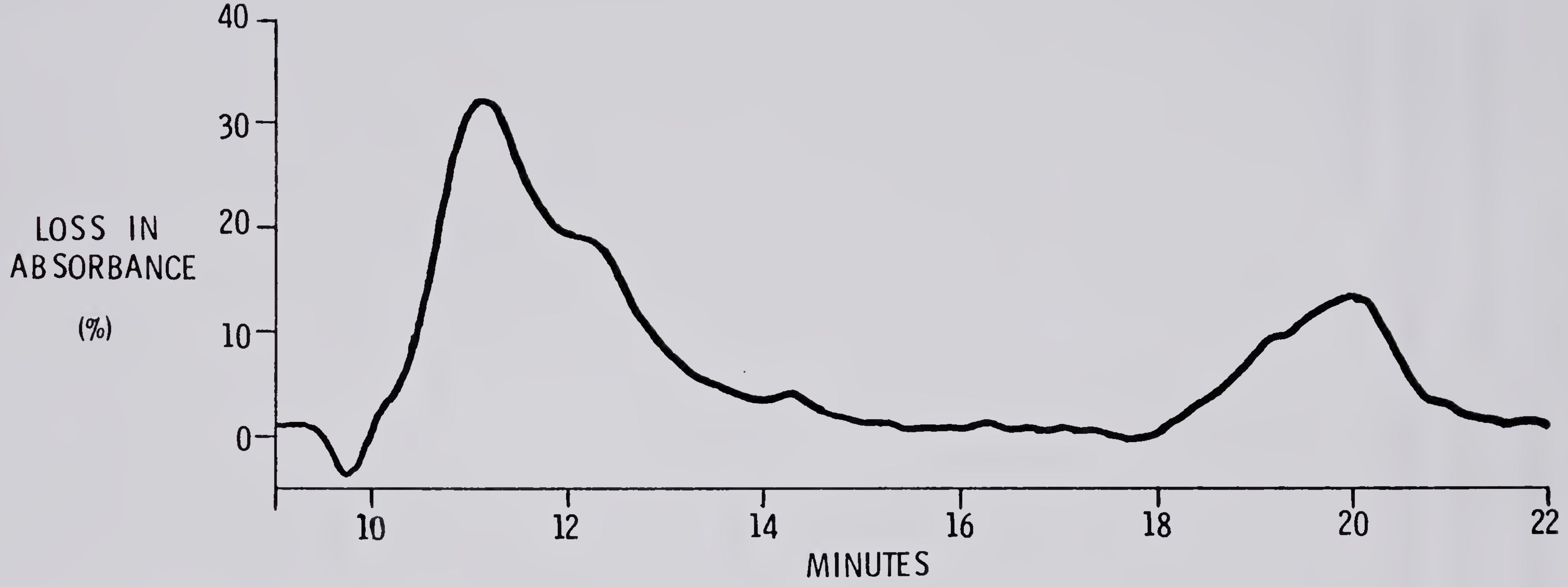


Figure 33. Automated non-digestive colorimetric analysis of soya lecithin extract using System II. Solvent system, acetonitrile:methanol:water (51:35:14 by vol.); flow rate 2.10 ml/min., room temperature, detection-colorimeter 660 nm cutoff filter, standard calibration 40% on inverse, column; Whatman silica gel 10 μ (500 x 4.6 mm). Injection of 900 μg.

[illegible]

workable system III. Unfortunately the response was only high for PC using the reduced molybdate reagent without acidification.

It is hoped that further development work on this reagent may lead to an effective automated system. This should be possible since the work presented in this thesis has led to a fuller understanding of the mechanisms involved in non-digestive colorimetric measurement.

CHAPTER V

CONCLUSIONS

HPLC SEPARATION

1. It is possible to separate most of the major phospholipids (PE, LPE, PC, Spm and LPC) using an acetonitrile:methanol:water (51:35:14 by vol.) solvent system on a silica gel column.

2. Ultraviolet detection after separation at 205 nm cannot be used for quantification because of the variation in absorbance due to the difference in extinction coefficients of phospholipids with different fatty acid side chains within each class.

NON-DIGESTIVE COLORIMETRIC ANALYSIS

1. The basis of non-digestive colorimetric analysis is the formation of an association complex between a molybdate compound and the phospholipid, which is then separated from excess reagent by extraction into a lipophilic solvent as a "molybdenum blue" chromophore.

2. Heating is not necessary for the complexation or the color development. Color development can be attained prior to association; thus this system is amenable to automation.

3. A number of different molybdenum blue compounds are extracted into lipophilic solvents after complexing with phospholipids.

4. Transfer of molybdenum complexes by phospholipid is dependent on the class and

fatty acid side chains of the phospholipid, acidity, temperature and the extracting solvent used.

5. Automation of non-digestive colorimetric analysis is possible; and further development is needed to make it an acceptable technique.

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APPENDIX I

PHOSPHOLIPID SOURCES

PL Biochemicals Inc., Milwaukee, Wis.

Phosphatidyl Choline (Bovine)	Lot 471-112
Lysophosphatidyl Ethanolamine (Bovine)	Lot 535-27
Lysophosphatidyl Choline (Bovine)	Lot 535-59
Phosphatidyl Ethanolamine (Bovine)	Lot 535-39
Phosphatidyl Inositol (Plant)	Lot 583-3
Phosphatidyl Serine (Porcine)	Lot 535-86
Sphingomyelin (Bovine)	Lot 471-31A

Sigma Chemical Company, St. Louis, Mo.

DL-3-Phosphatidyl Choline Dipalmitoyl (Synthetic 99%)	38C-0002
L-3-Phosphatidyl Choline Distearoyl (Synthetic 98%)	37C-8065
DL-3-Phosphatidyl Ethanolamine Dipalmitoyl (Synthetic)	96C-0090
Cardiolipin (DPG) (Bovine	57C-0017
L-3-Lysophosphatidyl Choline (Egg Lecithin Phospholipase A)	38C-8390
Sphingomyelin (Bovine)	96C-8086
Phosphatidyl Inositol (Crude Soy Bean 50%)	108C-8365
L-3-Lysophosphatidyl Ethanolamine (Egg Yolk)	58C-8381

Serdary Research Laboratories, London, Ontario

L-3-Phosphatidyl Choline Dilinolenoyl (Synthetic)	B-632
L-3-Phosphatidyl Choline Dioleoyl (Synthetic	B-63

Serdary Research Laboratories, London, Ontario

L-3-Phosphatidyl Choline Dilinoleoyl (Synthetic)	B-631
Lysophosphatidyl Choline, Dilinoleoyl (Synthetic)	B-64
Lysophosphatidyl Choline, Linoleoyl (Synthetic)	B-641

Non-commercial Source

Phosphatidyl Ethanolamine (Egg Yolk) (was a gift of F. Y. Shum from the W. W. Cross Cancer Institute, Edmonton).